UNIVERSITY OF ALBERTA

Active-site Probes for N-Acetylglucosaminyltransferase V

By

Dristee Ghoorohoo

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Master of Science

DEPARTMENT OF CHEMISTRY

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ABSTRACT

Previous work from this laboratory has shown that the trisaccharide octyl-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside **7** is a synthetic acceptor for GlcNAcT-V with a K_m of 23 μM. It has also been shown that the trisaccharide tolerates substitution at almost all positions but its activity is markedly decreased if there is substitution at the 3'', 4'' and 6'' hydroxyls groups of the terminal GlcNAc ring.

The syntheses of two analogs having a methoxy-group (**29**) and a hydrogen atom (**30**) respectively at the 2'' position of the GlcNAc residue were attempted, only the former succeeded. A third compound (**31**) incorporated a glucuronic residue instead of the GlcNAc ring. A simplified disaccharide (**32**) was prepared whereby a hydroxyethyl group replaced the mannose ring. Finally a control compound (**33**) was synthesized.

The compounds were then evaluated as substrates/inhibitors for human recombinant GlcNAcT-V. None of the compounds were substrates and only the methoxytrisaccharide showed a low but complex inhibitory activity.

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LIST OF ABBREVIATIONS

Å	Angstrom
Ac	acetyl
Ac_2O	acetic anhydride
AIBN	azobisisobutyronitrile
aq.	aqueous
Asn	asparagine
APT	attached proton test
Bn	benzyl
bs	broad singlet
BSA	bovine serum albumin
Bz	benzoyl
Calcd.	calculated
COSY	correlation spectroscopy
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DMAP	4-dimethylaminopyridine
DMF	N, N-dimethylformamide
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetate
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
ES	electrospray
FAC/MS	frontal affinity chromatography coupled to mass spectrometry
Fuc	fucose

Gal	galactose
GalNAc	2-acetamido-2-deoxy-D-galactopyranose
gem	geminal
Glc	glucose
GlcNAc	2-acetamido-2-deoxy-D-glucopyranose
GlcNAcT	N-acetylglucosaminyltransferase
h	hour(s)
HMQC	inverse 2D ^{1}H-^{13}C heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
Hz	Hertz
J	coupling constant
K_i	inhibitory constant
K_m	Michaelis constant
m	multiplet
m/z	mass to charge ratio
Man	mannose
Me	methyl
mg	milligram(s)
MHz	megahertz
min	minute(s)
mL	milliliter(s)
MMT	mono-(p-methoxy)-trityl
mol	mole(s)
mmol	millimole(s)
MS	mass spectrometry
NDP	nucleoside diphosphate
NMR	nuclear magnetic resonance

Phth	phthaloyl
ppm	parts per million
Py	pyridine
q	quartet
RER	rough endoplasmic reticulum
r.t	room temperature
S.A	sialic acid
t	triplet
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
TOCSY	total correlation spectroscopy
UDP	uridine diphosphate
vic	viccinal
V_{max}	maximum velocity of enzyme catalyzed reaction

Chapter 1

Introduction

1.1 General Overview

Oligosaccharides play important roles in diverse biological processes such as generating energy, as signaling molecules or as structural components. This latter role of carbohydrates is of utmost importance in the construction of multicellular organisms, which requires interaction between cells and the surrounding matrix[1]. Actually all animal cell surfaces are covered with a dense array of covalently attached sugar chains[2,3] called glycans. These glycans are very important in modulating and mediating a variety of cellular events crucial to the development of an organism. A few examples include the binding of viruses[4-6] and bacteria[7,8], cell-cell recognition[9], the binding of the sperm to the egg during fertilization[10] and guiding neuronal development[11,12].

Interestingly carbohydrates also play an important role in the development of cancerous tissues[13-18] and recently many diseases have been correlated with changes in the structures of glycoconjugates[1,19-25]. Therefore this has led to a resurgence of carbohydrate chemistry and in 1988, Rademacher, Parekh and Dwek first coined the term "Glycobiology". Glycobiology is the study area that looks at the cellular and molecular biology of glycans and today it is one of the fastest growing fields in the biomedical sciences. The increased interest and therefore research being done in this field will definitely help us to both understand better the nature of diseases and to design carbohydrate-based therapeutics.

1.2 Cell Surface Carbohydrates

The three main classes of carbohydrates found on the cell surfaces of animal cells

are glycoprotein, glycolipid and proteoglycan. Glycoproteins are compounds in which a protein carries one or more oligosaccharide chains covalently attached to a polypeptide backbone. Fig. 1 shows a typical cell surface membrane covered with glycoproteins[26]. These carbohydrates can be a single saccharide unit or a long polysaccharide chain. There are two major classes of glycoprotein, namely O-linked glycoproteins and N-linked glycoproteins, and they will be discussed in more detail in section 1.2.1 and 1.2.2 respectively. Glycolipids are compounds where lipids embedded in cell membranes have carbohydrate chains attached to them. Lastly, proteoglycans are chains of acidic and unbranched carbohydrates units which are often sulphated.

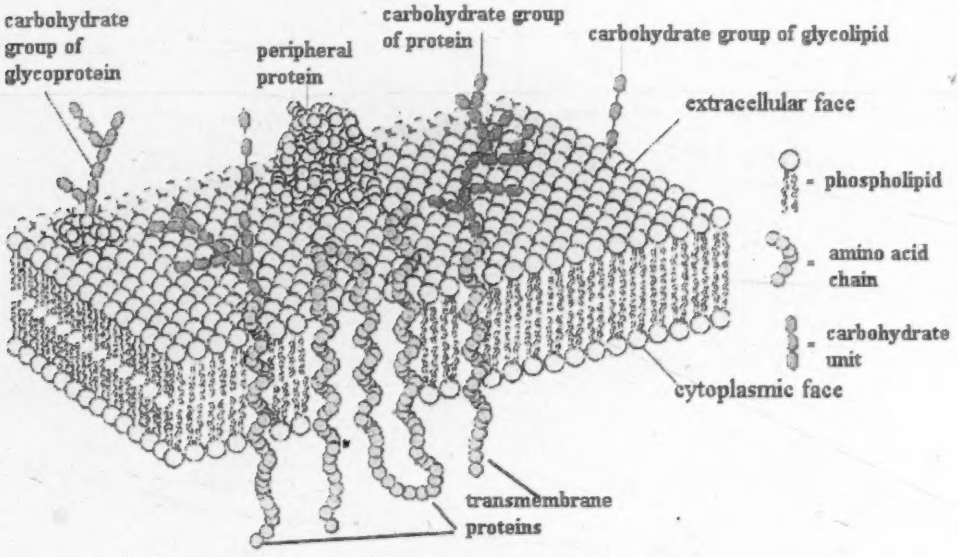


Fig. 1: A typical cell surface membrane covered with glycoproteins[26]

1.2.1 O-Glycans

O-Linked oligosaccharides are formed from the addition of an N-acetylgalactosamine residue from uridine diphosphate N-acetylgalactosamine (UDP-GalNAc) to serine/threonine residues on proteins. Fig. 2 represents a simple example of an O-glycan structure where the α-GalNAc is bound to serine. This addition reaction is catalyzed by N-acetylgalactosaminyltransferase (GalNAcT). The product can then be

elongated by Gal and GlcNAc residues and may be terminated by Gal, GalNAc, GlcNAc, Fuc, sialic acid residues and sulfate esters[27]. Each residue is added sequentially by the action of glycosyltransferases in the Golgi apparatus. Note that in some cases only one N-acetylneuraminic acid (sialic acid) may be present. O-Glycans are usually less branched than N-glycans and are more often biantennary structures. Clusters of O-glycans on cell surfaces or secreted glycoproteins are usually termed as mucin[28].

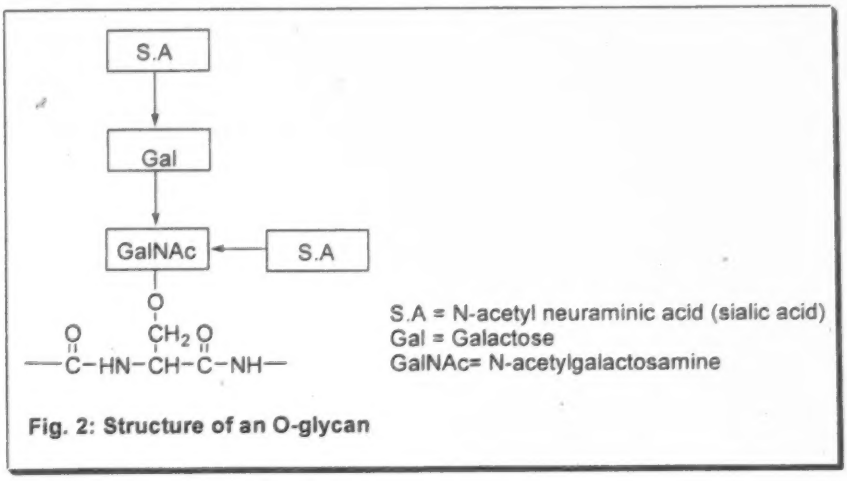


Fig. 2: Structure of an O-glycan

1.2.2 N-Glycans

The other type of glycoprotein contains N- or asparagine-linked oligosaccharides. In this class of glycoprotein, carbohydrate chains are bound to asparagine in the sequence Asn-X-Ser/Thr where X can be any amino acid except proline[29]. These glycoproteins usually consist of 1-20% carbohydrate by weight which represents 1-10 oligosaccharides attached to the protein backbone[30]. They are either membrane bound or soluble products of a biosynthetic system found in the secretory pathway of eukaryotic cells.

These glycoproteins are diverse in structure but they all have a common core structure consisting of the pentasaccharide, Manα(1→6)[Manα(1→3)]Manβ(1→4)GlcNAcβ(1→4)GlcNAc-Asn. Fig. 3 shows the core structure common to all N-linked glycoproteins.

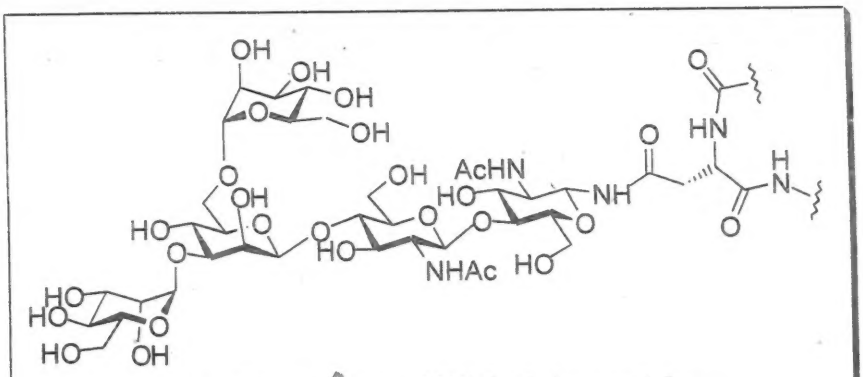


Fig. 3: Core structure of N-linked glycoproteins

Extracellular N-glycans in vertebrates exist as high mannose, hybrid or complex subtypes. Fig. 4 shows the three major classes of asparagine-linked oligosaccharides. The core pentasaccharide common to all N-linked structures is enclosed in the box.

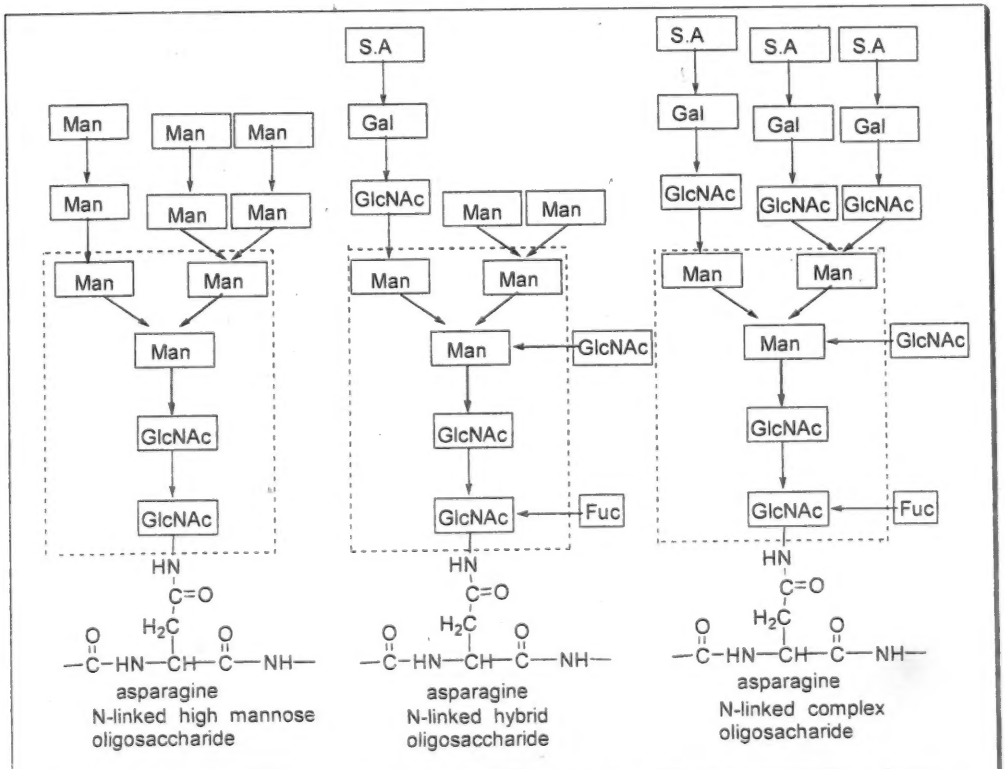


Fig. 4: Different classes of N-linked oligosaccharides[31]

In the high-mannose subtype (Fig 4), the core pentasaccharide structure terminates with unsubstituted mannose residues. They typically have two to six additional mannose residues linked to the pentasaccharide core. The hybrid subtype has features from both high mannose complex-type oligosaccharides. The pentasaccharide core ends with both substituted (with GlcNAc linkage) and unsubstituted mannose residues. Most hybrid N-glycans have a bisecting N-acetylglucosamine linked β1,4 to the β-mannose of the core pentasaccharide. The complex-type oligosaccharides are those glycans in which both the α3- and α6-linked mannose residues of the pentasaccharide core are substituted with GlcNAc moieties. The bisecting N-acetylglucosamine linked β1,4 to the β-mannose of the pentasaccharide core is usually present. Moreover, the innermost N-acetylglucosamine residue can be linked to an α1,6 fucose residue. Usually, the terminal GlcNAc residues carry a galactose followed by sialic acid moieties. This complex oligosaccharide can have additional branches on the α-mannose residues or the outer chains can be elongated by the addition of other sugar residues such as fucose, sialic acid or galactose. Most complex oligosaccharides have 2-4 outer branches though five outer branches have also been observed[31]. Analysis of total N-glycan from various cells has shown that most vertebrate extracellular N-glycans are of the complex subtype. The functions of the carbohydrate chains on these glycoproteins include a role in the folding of the protein, an important component of ligand-receptor interaction, affecting solubility and the three-dimensional structure[1,32].

1.3 Biosynthesis and Processing of N-Oligosaccharides

Studies on glycans have been lagging behind mainly because of their complexities compared to other classes of compounds[33]. Unlike nucleotide and protein, which are linear polymers with only one basic type of linkage, monosaccharide can generate two types of linkages, α or β, to any one of several positions on another monosaccharide giving rise to branches. Therefore the biosynthesis of glycans cannot be

controlled by a template approach but requires an assembly line. Two classes of enzymes, glycosidases and glycosyltransferases are involved in the biosynthesis of N-glycans. Glycosidases are enzymes which cleave glycosidic linkages[34] and they can be classified into catabolic enzymes and trimming enzymes. Catabolic glycosidases are those which catalyze the breakdown of ingested oligo- and polysaccharides, or carbohydrates of glycoproteins and glycolipids. During glycoprotein biosynthesis, trimming glycosidases are involved in the processing of carbohydrate chains. Glycosyltransferases catalyze the transfer of a monosaccharide from a glycosyl donor nucleotide to the hydroxyl group on a glycosyl acceptor in a regio- and stereospecific manner[33]. This latter class of enzymes will be discussed in more detail in section 1.4.

All eukaryotic cells have conserved the earlier steps in the biosynthesis of N-glycans as well as some subsequent processing reactions. The common core unit found in N-linked glycoproteins suggests that all N-linked glycoproteins are formed from a common precursor. Subsequently, specific N-glycoproteins are formed by the sequential addition and removal of saccharide residues to the core unit. All these processes occur in the two cellular organelles, the endoplasmic reticulum (ER) and the Golgi apparatus.

1.3.1 Assembly of the Dolichol Pyrophosphoryl Oligosaccharide

The first step in the biosynthesis of N-linked oligosaccharides is the formation of a large precursor oligosaccharide referred to as the dolichol pyrophosphoryl oligosaccharide. The structure of dolichol pyrophosphoryl oligosaccharide is shown in Fig. 5. The assembly of this oligosaccharide precursor occurs on both faces of the ER[35]. The oligosaccharide portion of this dolichol pyrophosphoryl oligosaccharide consists of three glucose, nine mannose and two N-acetylglucosamine molecules. This is then linked by a pyrophosphoryl residue to dolichol, a long-chain of polyisoprenoid lipid.

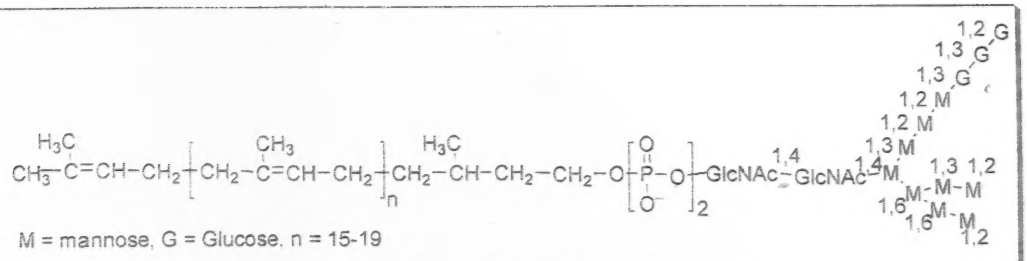


Fig. 5: Structure of dolichol pyrophosphoryl oligosaccharide

This precursor oligosaccharide is formed by a series of reactions using membrane bound enzymes of the rough endoplasmic reticulum (RER) where each sugar residue is added one at a time to dolichol phosphate. The formation of the dolichol pyrophosphoryl oligosaccharide precursor in the ER is shown in Fig. 6. Since the concentration of these enzymes is low and they are membrane bound, they have not been studied as purified enzymes. Therefore, information about the reactions they catalyze has been obtained from genetics, use of inhibitors and *in vitro* reactions using membrane preparations.

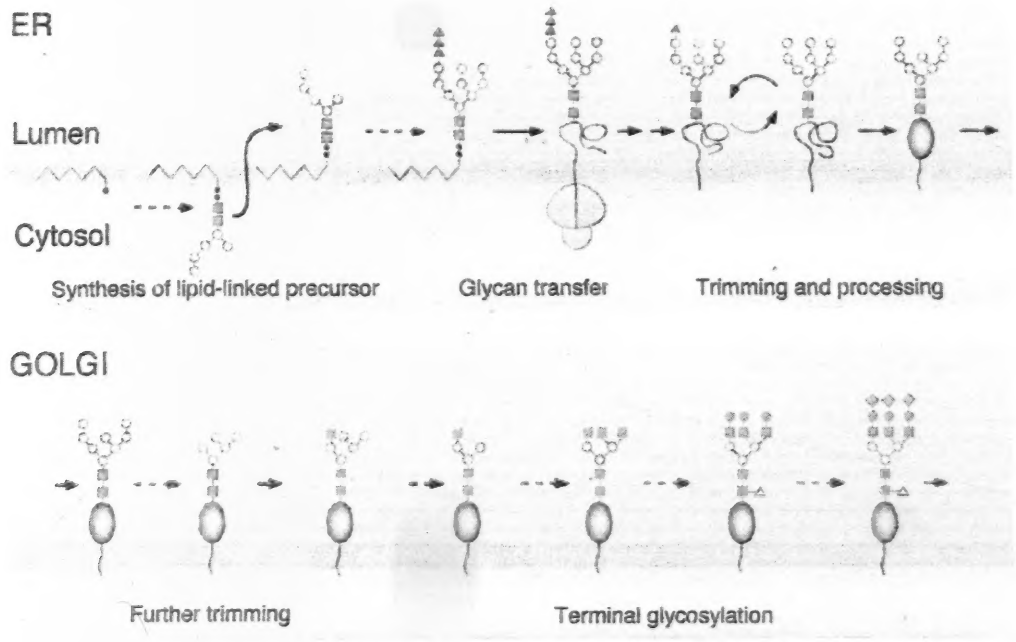


Fig. 6: Assembly of the dolichol pyrophosphoryl oligosaccharide, its transfer to asparagine on nascent protein and processing of N-linked glycoproteins[42]

The first step involves the addition of GlcNAc-P from UDP-GlcNAc to dolichyl phosphate in the presence of GlcNAc-1-phosphotransferase (L-G1PT). This results in the formation of dolichol-P-P-GlcNAc. L-G1PT enzyme is a hydrophobic protein with its active site on the cytosolic face of the ER[36]. This enzyme is inhibited by a fungal antibiotic, tunicamycin (TM), which is specific to this initial enzyme in the assembly pathway[37]. Studies using tunicamycin as inhibitors have shown that cells die by apoptosis implying that glycosylation is essential for cell survival[38]. The following six steps involve the successive addition of a GlcNAc and five mannose residues, each catalyzed by membrane-bound transferases, to dolichol-P-P-GlcNAc. Labeling experiments using [2-^{3}H]Man have shown that there is no accumulation of intermediates having structures smaller than dolichol-P-P-$(GlcNAc)_2(Man)_5$ in this pathway[39]. Removal of the genes encoding for these enzymes is fatal.

The dolichol-P-P-$(GlcNAc)_2(Man)_5$ flips across the ER membrane and four mannose residues are added to form dolichol-P-P-$(GlcNAc)_2(Man)_9$. These reactions are catalyzed by membrane bound mannosyltransferases which use Dol-P-Man as sugar donor[39]. These enzymes are not essential for cell survival. Finally, three glucose residues are added by three different membrane bound glucosyltransferase enzymes from Dol-P-Glc. Both Dol-P-M and Dol-P-Glc are formed on the cytoplasmic side of the ER and then flipped across the lipid bilayer. Absence of these enzymes does not result in cell death. It was believed that the presence of the glucose residues is crucial for the transfer of the oligosaccharide from dolichol pyrophosphoryl oligosaccharide to protein[40]. However, it has been shown that non-glucosylated $(GlcNAc)_2(Man)_9$ is transferred only slightly slower (less than two-fold) than the glucosylated one[41]. Therefore the function of the glucose residues is not clear but it may be associated in regulating the level of oligosaccharyl dolichol. The oligosaccharide dolichol is positioned in such a way that the dolichol portion is embedded in the rough ER membrane and the sugar residues face the lumen of the organelle.

1.3.2 Transfer of the Oligosaccharide from Carrier Lipid to Asparagine

Once the dolichol linked high-mannose oligosaccharide is formed, it is transferred *en bloc* onto an asparagine residue on nascently translated polypeptide by the enzyme oligosaccharyltransferase complex (OST). Fig. 6 represents a schematic diagram of the transfer reaction. Note that the oligosaccharide is transferred as soon as the asparagine crosses the luminal membrane. During the reaction the high-energy GlcNAc-P bond is cleaved releasing dolichyl pyrophosphate which is hydrolyzed to generate dolichol phosphate[43]. The level of dolichol phosphate might serve as a regulator for the assembly of dolichol oligosaccharide. The asparagine should be in the tripeptide recognition sequence Asn-X-Ser/Thr (where X is any amino acid except proline)[29,44]. However, it is worthy to note that not all Asn-X-Ser/Thr sequons are glycosylated. Therefore in order to investigate this statement, in 1998 Mellquist *et al.*[29] studied the effect of the amino acid (Y) following on Asn-X-Ser/Thr sequon (*e.g* Asn-X-Ser/Thr-Y). Amino acids near the tripeptide sequence can effectively affect glycosylation by influencing conformation of the sequon or by participating in hydrogen bonding. As models for the tripeptide, they used Asn-Leu-Ser and Asn-Leu-Thr each followed by the 20 common amino acids at the Y position. They found out that indeed different amino acids at the Y position influence the efficiency of glycosylation. The effect was markedly greater for the Asn-Leu-Ser sequence. As expected, proline was found to have an inhibitory effect on glycosylation for both Ser and Thr tripeptide sequence. It is suggested that peptides containing proline adopt a conformation that is incompatible with recognition or catalysis by the enzyme. Cysteine was also found to be inhibitory when there was the potential for neighboring disulfide bridges between cysteines residues. The large group on Trp also blocks accessibility for the transfer reaction. The influence of charged amino acid is more complex as Glu impairs glycosylation while Asp has no effect. Interestingly Thr, Ser and Cys have favorable effects suggesting that they might be involved in hydrogen bonding with amino acids in enzyme active site helping in the transfer reaction. Sequons near the

C-terminal of the peptide chains are usually not glycosylated because ongoing folding of the protein imposes some conformational constraints. However, it is useful to consider that other factors for *e.g.* level of oligosaccharyltransferase, dolichol phosphate[45], dolichol-P-P-oligosaccharide and rate of protein translation into ER can also influence glycosylation.

Around two decades ago, the main biochemical properties of the oligosaccharyltransferase enzyme were determined using intact or detergent solubilized microsomal membrane preparations as a source of enzyme. It is a hetero-oligomeric membrane bound enzyme. The optimum pH for the enzyme is between 6.5 and 7.5 and it requires a divalent metal cation (*e.g.* Mn^{2+}) for catalysis[46]. Previous attempts to purify the enzyme have failed because of the instability of the enzyme upon solubilization. However, recently this problem was solved when it was found that the enzyme is stable when phosphatidyl choline is added in purification buffers. This has resulted in the purification of the enzyme from various sources[47,48] and since then significant research has been done on this enzyme[44,49]. The mechanism for the transfer reaction has been proposed by Imperiali *et al.*[50,51]. The postulated mechanism of the reaction is shown in Fig. 7. In her proposed reaction mechanism, the peptide backbone adopts an Asx-turn conformation. The β-carbonyl group of the asparagine residue interacts with both the backbone amide and the hydroxyl group on the Ser/Thr by hydrogen bonding. A basic residue, present in the active site of the enzyme, deprotonates the asparagine nitrogen generating a nucleophilic species which could react with the electrophilic linked oligosaccharide.

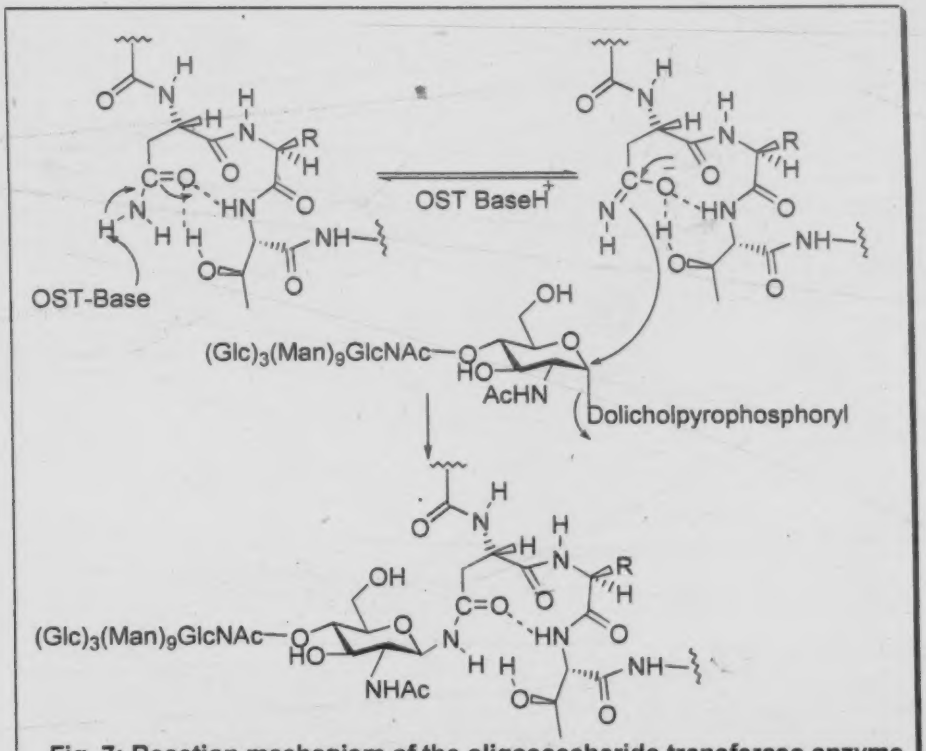


Fig. 7: Reaction mechanism of the oligosaccharide transferase enzyme

1.3.3 Processing and Trimming of Asparagine-Linked Glycoproteins

Immediately after the oligosaccharide is transferred to the polypeptide, glucosidase I specifically removes the terminal α-1,2-glucose residue which is followed by the action of glucosidase II which rapidly removes the first α-1,3-glucose residue[52]. It takes a little longer to remove the innermost glucose residue and this has been associated with a protein folding mechanism[53]. In 1993, Ou *et al.* discovered calnexin, an ER transmembrane protein, which binds with incompletely folded glycoproteins[54]. Fig. 8 shows the pathway of calnexin association to glycoproteins. Shortly afterwards, Hammond *et al.* found that monoglucosylated proteins were the most likely binding species to calnexin[55]. Furthermore they observed that association to calnexin did not occur when cells were treated with either tunicamycin or inhibitors of glucosidase I and II (castanospermine and 1-deoxynojirimycin, respectively). Finally in 1995, Hebert[56]

proposed that calnexin association occurs only to monoglucosylated core glycans which can originate either after the removal of one glucose by glucosidase II or by reglucosylation of already misfolded unglucosylated high mannose glycans. A second lectin, calreticulin, appears to promote proper folding of N-glycans[53].

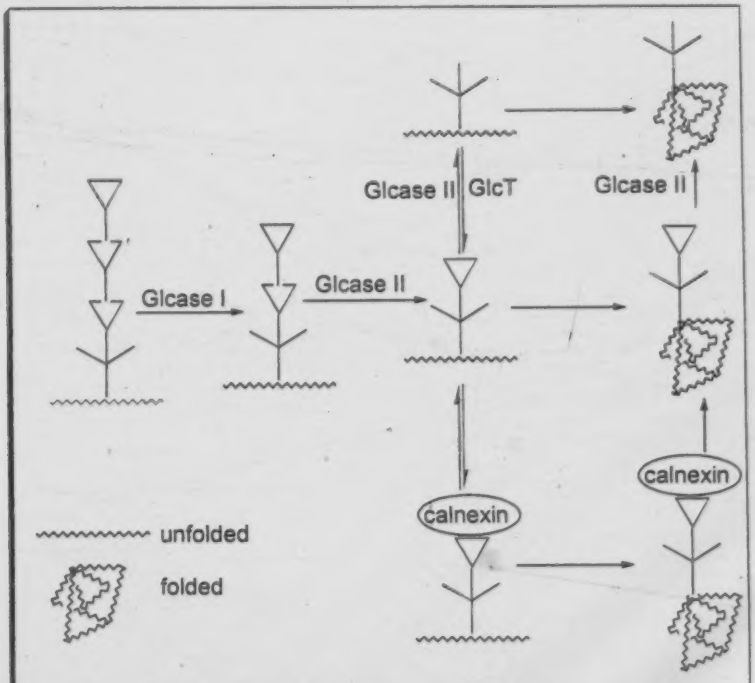


Fig. 8: Calnexin mediated folding of glycoprotein[56]

Finally α-1,2 mannosidase catalyzes the removal of one particular α-1,2 mannose sugar and the glycoprotein $(Man)_8(GlcNAc)_2$ is then transported in vesicles to the cis face of the Golgi apparatus. Here the glycoprotein is processed according to its final destination resulting in high mannose, hybrid or complex subtypes[31]. Fig. 9 shows the processing of N-linked glycoproteins to form the high mannose, hybrid and complex oligosaccharide. A series of reactions occur during the processing of glycoproteins in the Golgi apparatus[57]. α-Mannosidase I catalyzes the removal of mannose residues from $(Man)_8(GlcNAc)_2$ to form structures having 5-7 mannose residues. $(Man)_8(GlcNAc)_2$

can also be present if the glycoproteins is buried inside the protein structure so that it is not accessible to the enzyme.

N-acetylglucosaminyltransferase I (GlcNAcT-I) catalyzes the addition of β1,2 N-acetylglucosamine to $(GlcNAc)_2(Man)_5$ to form a hybrid structure $(GlcNAc)_2Man(GlcNAc)$. Hybrid oligosaccharides are formed if the enzyme α-mannosidase II cannot act on $(GlcNAc)_2(Man)_5(GlcNAc)$ to remove the two outer mannose residues (reaction 3, Fig. 9). Therefore only the branch containing the GlcNAc undergoes elongation by addition of galactose followed by sialic acid. Fucose can also be added to an N-acetylglucosamine residue linked to asparagine in the presence of fucosyltransferase. Core fucosylation is found on hybrid and complex N-glycans but is inhibited by bisecting GlcNAc added by GlcNAcT-III. Lastly, for complex oligosaccharides, two more mannose residues are removed by mannosidase II from $(GlcNAc)_2(Man)_5(GlcNAc)$ in the medial Golgi apparatus. Two more N-acetylglucosamine residues are added. Subsequent addition of galactose and sialic acids on the three branches (antennae) result in formation of multi-antennary polylactosamine. The final structure of the glycoprotein differs depending on the order in which competing enzymes act on the same substrate. It has been shown that some glycosyltransferases compete for identical substrate, and products may inhibit the action of other glycosyltransferases. Some interesting observations during glycosylation are as follows:

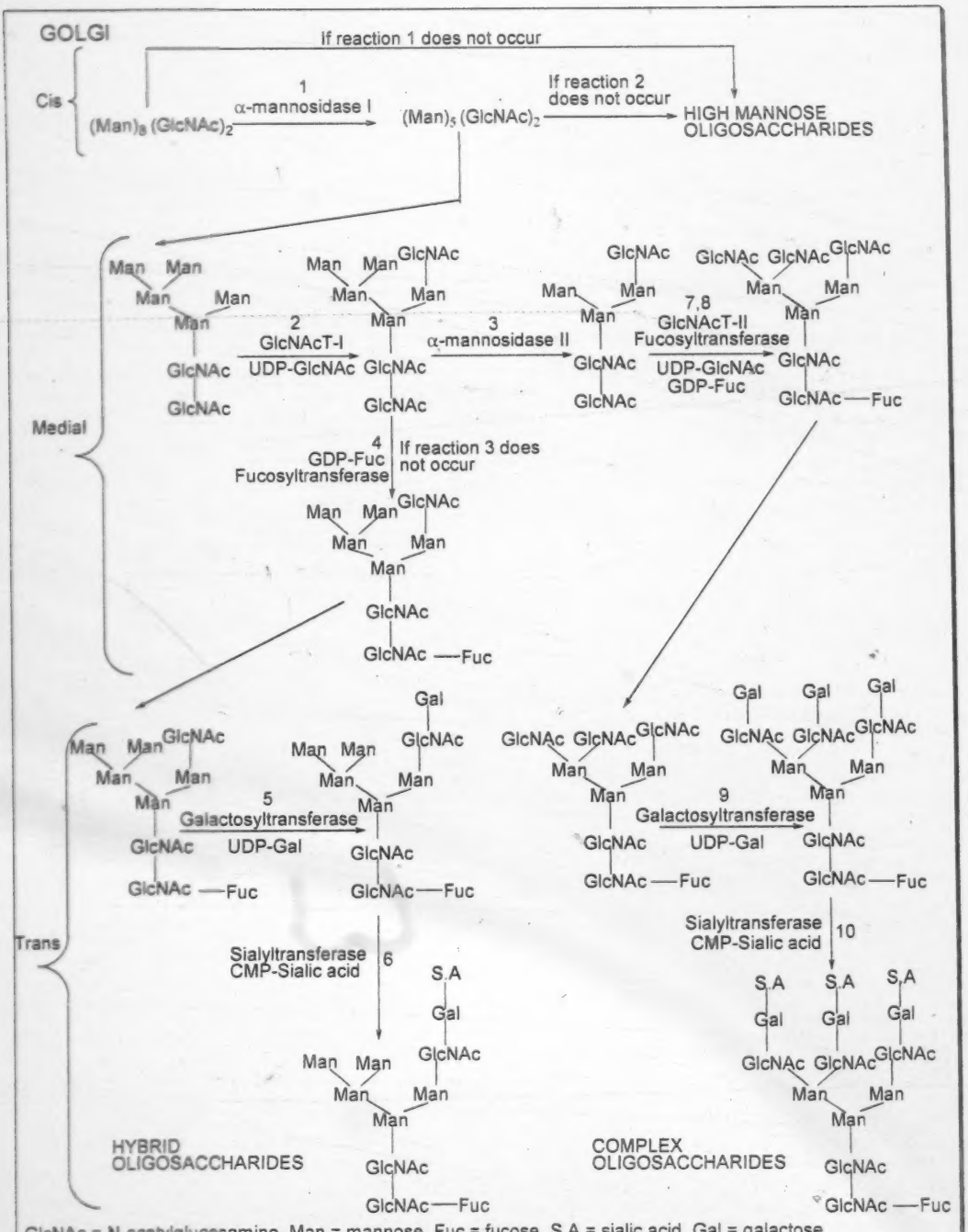
(i) α-Mannosidase II cannot cleave the two outer mannose residues after GlcNAcT-III has acted on the hybrid $(GlcNAc)_2(Man)_5(GlcNAc)Asn$.

(ii) Prior activity of GlcNAcT-III inhibits GlcNAcT-IV and GlcNAcT-V branching.

(iii) Prior activity of both GlcNAcT-I and GlcNAcT-II is required for GlcNAcT-V activity.

(iv) Terminal branches containing both fucose and sialic acid cannot be formed[57].

The processed glycoproteins are then transported to their final destination either inside the cell or on cell surfaces with the oligosaccharide facing the outside of the cell.



GlcNAc = N-acetylglucosamine, Man = mannose, Fuc = fucose, S.A = sialic acid, Gal = galactose
UDP = uridine diphosphate, CMP = cytidine monophosphate, GDP = guanosine diphosphate

Fig. 9: Processing of N-linked oligosaccharides in the Golgi apparatus

1.4 Glycosyltransferases

Glycosyltransferases catalyze the transfer of a sugar residue from an activated glycosyl donor (usually sugar-nucleotide) to the hydroxyl group of a glycosyl acceptor in a regio and stereospecific manner[33]. These enzymes are usually very specific for both substrates. With few exceptions, there is at least one enzyme for every glycosidic linkage found in nature. Therefore it is estimated that several hundred glycosyltransferases must exist for the synthesis of diverse carbohydrate structures in glycoproteins and glycolipids. There are two kinds of glycosyltransferases, namely Leloir and non-Leloir, based on the glycosyl donors they use[58]. Non-Leloir glycosyltransferases are those that usually use glycosyl phosphates as donors while Leloir glycosyltransferases use sugar nucleotides as donors. The latter can be further classified as retaining enzymes or inverting enzymes, depending on the configuration of the newly formed linkage. Fig. 10 illustrates the two ways by which Leloir glycosyltransferases can transfer a monosaccharide to an acceptor. During the biosynthesis of mammalian glycoproteins, most glycosyltransferases are the Leloir types. Glycosyltransferases are grouped into families according to the type of sugar they transfer and each enzyme has a specific name depending on the type of glycosidic linkage formed (α or β) and the specific hydroxyl group in the acceptor where the transfer occurs. Mammalian glycosyltransferases utilize nine main sugar nucleotides as building blocks to construct complex oligosaccharides. They can be grouped into activated sugar donors of uridine diphosphate (UDP-Gal, UDP-GalNAc, UDP-Glc, UDP-GlcNAc, UDP-Xyl, UDP-GlcA), guanosine diphosphate (GDP-Fuc, GDP-Man) and cytidine monophosphate (CMP-Sialic acid/CMP-Neu5Ac). With the exception of fucose, which is in the L configuration, all the sugar units have the D configuration.

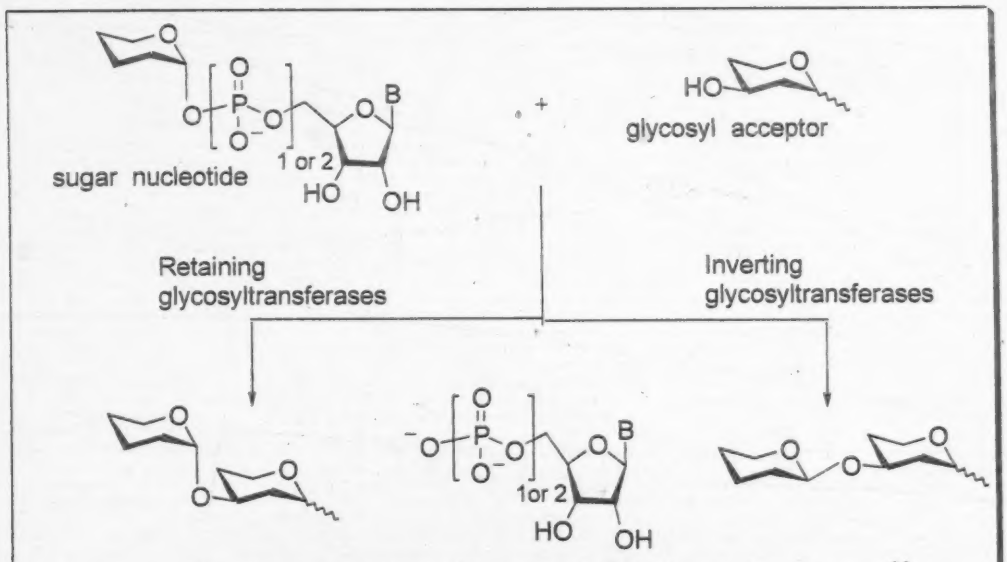


Fig. 10: Reactions catalyzed by Leloir glycosyltranferases[33]

Glycosyltransferases are usually membrane-bound proteins of the endoplasmic reticulum and the Golgi apparatus. For *in vitro* experiments using glycosyltransferases, lysis of the membrane with the aid of detergents is required to liberate and express the activity of the enzymes. With the progress in molecular biology and biotechnology, more than 100 glycosyltransferases from viral[59], bacterial[60,61] and mammalian[62,63] sources have been cloned. Analysis and comparison of the amino acid sequences show that there is practically no sequence homology between families of glycosyltransferases which have similar donors or acceptors as substrates while a high degree of conservation (~80%) exists between glycosyltransferases from widely divergent species. However, amino acid sequences of terminal glycosyltransferases located in the medial and trans Golgi apparatus predict that these enzymes have a characteristic topology of the so-called type II transmembrane proteins. Fig. 11 depicts the common topography of Golgi glycosyltransferases. It consists of a short NH_2 terminal cytoplasmic tail, a 16-20 amino acid hydrophobic signal-anchor domain that spans the membrane, an extended stem

region and a large COOH-terminal catalytic domain oriented within the lumen of the Golgi cisternae[64]. The stem region serves as a flexible tether allowing the catalytic domain to bind and glycosylate the carbohydrate groups of proteins. Sometimes proteolytic enzymes can cleave the stem region to liberate catalytically active soluble forms of the enzyme that may be released from the cell[65].

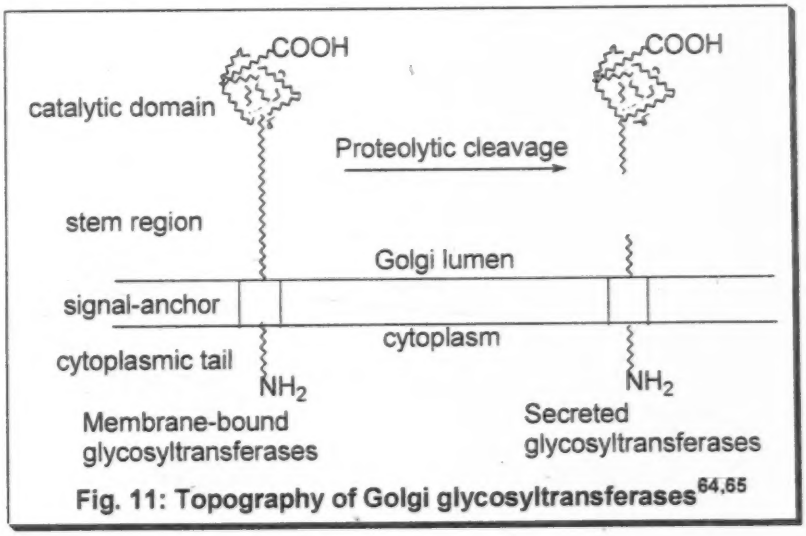


Fig. 11: Topography of Golgi glycosyltransferases[64,65]

Most glycosyltransferases require the presence of a divalent metal cation (Mg^{2+}, Mn^{2+}) as a cofactor during the glycosylation reaction. The divalent cation is thought to participate in glycosyl activation by coordination to the pyrophosphate oxygen[66]. The enzymes are most effective in the pH range of 5.0 to 7.0, which is the typical pH values found in different parts of the ER and Golgi apparatus. Usually glycosyltransferases act in a sequential manner such that the product of one glycosyltransferase reaction becomes the substrate for the subsequent action of another glycosyltransferase enzyme. This results in a linear or branched polymer of monosaccharides linked to each other[31].

Two mechanisms have been put forward to explain the two types of glycosyltransferase reaction. Fig. 12 illustrates the mechanism for a glycosyltransferase

reaction with inversion of configuration. This type of reaction involves direct S_N2 displacement of the nucleoside diphosphate from the sugar nucleotide by the hydroxyl group of the acceptor. Since the hydroxyl group is not very nucleophilic, it has been suggested that it is activated by coordination to a basic group present in the active site of the enzyme.

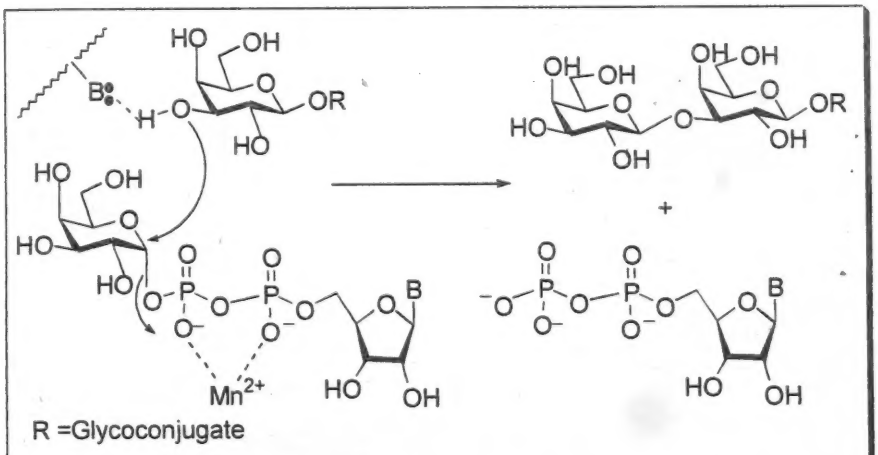


Fig. 12: Proposed mechanism for a glycosyltransferase reaction with inversion of configuration

For the retention glycosylation reaction, it is proposed that it occurs by a double displacement reaction[67](Fig. 13). First of all, a nucleophile present in the active site of the enzyme reacts to become attached at the anomeric center of the sugar nucleotide donor displacing the nucleotide diphosphate (NDP). This is then followed by the nucleophilic displacement of the enzyme from the glycosyl-enzyme intermediate by the hydroxyl group of the acceptor.

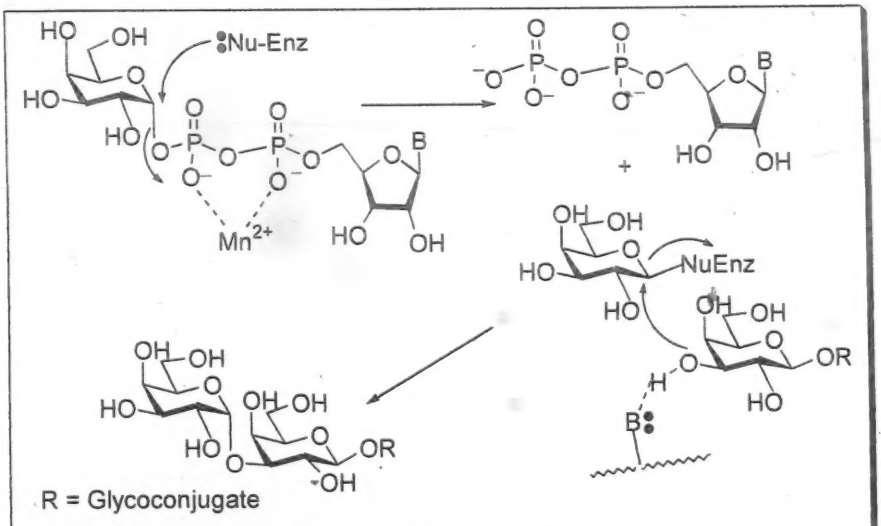


Fig. 13: Proposed mechanism for a glycosyltransferase reaction with retention of configuration

1.5 N-Acetylglucosaminyltransferase V (GlcNAcT-V)

N-acetylglucosaminyltransferase V (EC number: 2.4.1.155) is an enzyme in the N-acetylglucosaminyltransferase family. There are actually six enzymes in this family and they add a GlcNAc residue to the core pentasaccharide of N-glycans and after elongation with groups like galactose, sialic acids and fucose, bisected multi-antennary glycans are formed. The position where each of these enzymes adds a GlcNAc residue is shown in Fig. 14. GlcNAcT-V adds a GlcNAc residue in a β1-6 linkage to the Manα1-6Manβ of the core N-glycan. Prior action of GlcNAcT-I and GlcNAcT-II are needed for the action of GlcNAcT-V while the action of GlcNAcT-III inhibits its action[68]. It is the only GlcNAc transferase enzyme in the N-linked pathway that does not need the presence of a divalent cation[69].

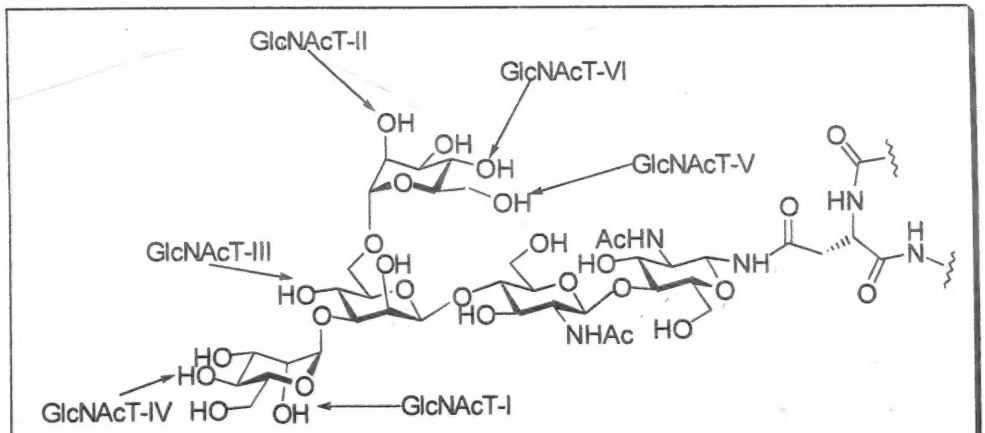


Fig. 14: Core structure of N-linked glycoproteins

In 1992, Shoreibah *et al.*[69] purified GlcNAcT-V from rat kidney by sequential affinity chromatography using UDP-hexanolamine-agarose followed by a synthetic inhibitor Agarose column. The total yield was 26% with an increase of 450,000 fold in purity. One year later, the gene encoding GlcNAcT-V was cloned by the same group[70]. An antiserum to recombinant, soluble GlcNAcT-V was prepared and it was used to visualize the enzyme in situ in baby hamster kidney cells using immunofluorescence microscopy[71]. Similar advances have been made on the human GlcNAcT-V, which was purified by an affinity column chromatography using the substrate as ligand from human lung cancer cell line[72]. A human GlcNAcT-V cDNA clone was obtained from a human fetal liver library in 1994[73]. Finally the Chinese hamster gene was also cloned by J.Weinstein *et al.*[74]

Further studies on human GlcNAcT-V showed that it has 741 amino acids and it is assumed to have six N-glycosylation sites. High homology (97%) at the amino acid level exists between human and rat GlcNAcT-V with one amino acid (valine) insertion in human GlcNAcT-V. GlcNAcT-V, which contains 17 exons and spans 155 kilobase pairs, is expressed in a tissue- and cell type-specific manner and is regulated at the level of transcription by multiple promoters[75]. Kang *et al.* demonstrated that the transcriptional

regulation of the GlcNAcT-V gene was mediated by the transcriptional factor ets-1. They arrived at this conclusion after doing some mutation analysis and cotransfection assaying on a human bile duct carcinoma cell line[76]. Further work on the relation between GlcNAcT-V and ets-1 showed that ets-1 plays a significant role in regulating the expression of GlcNAcT-V in a variety of cancers and might thus determine the potential for malignancy *via* the action of GlcNAcT-V[77]. The ets family shares a highly conserved DNA binding domain that interacts specifically with sequences that contain a common core trinucleotide sequence GGA. These studies provide possible insights into tumor metastasis *via* the regulation of GlcNAcT-V by ets-1.

1.6 GlcNAcT-V Associated with Metastasis

Increased poly-N-acetyllactosamine sequence [GlcNAc-β(1,3)Gal-β(1,4)], expressed on tri- and tetraantennary N-linked glycans due to increased β1-6 branching, has been seen in several malignant cell types including baby hamster kidney cells transformed by polyoma[78] (a DNA papovirus) and Rous sarcoma[79] (an RNA retrovirus) viruses or rat fibroblast cells in which oncogenes were activated[80]. Moreover, the activity of GlcNAcT-V in non-metastatic murine mammary carcinoma strongly correlates with acquisition of metastatic protential[16].

A completely novel strategy[81] to suppress lung metastasis of mouse melanoma was attempted by Yoshimura *et al.* Clones containing high activity of N-acetyl glucosaminyltransferase III (GlcNAcT-III) were intraveneously injected in mice and this resulted in significantly suppressed metastasis due to competition between GlcNAcT-III and GlcNAcT-V. Furthermore GlcNAcT-V does not act on bisected N-glycans. Invasion and cell attachment, in the extravasation stage of metastasis, was also inhibited on the GlcNAcT-III transfectants. Pierce *et al.*[15] showed that mouse melanoma cells cultured in different concentrations of retinoic acid had decreasing activity of GlcNAcT-V to increasing levels of retinoic acid. The number of cells per culture also decreased in

the same trend with increasing concentrations of retinoic acid. Further experiments demonstrated that these effects were due to a reduction in mRNA for GlcNAcT-V. An interesting observation made by Demetriou *et al.*[13] was that mice deficient in GlcNAcT-V showed kidney autoimmune disease and dysregulation of this enzyme in humans may increase susceptibility to autoimmune diseases such as multiple sclerosis.

GlcNAcT-V displays a prometastatic effect, which can be ascribed to stabilization of active matriptase by addition of a β1-6 GlcNAc side chain[18]. Matriptase is a type II, integral membrane serine protease, which may be involved in tissue remodeling, cell growth and cancer metastasis. It accomplishes the latter role by activating two important cancer invasion effectors on the surface of cancer cells. An increase in metastasis to the lymph nodes was observed following peritoneal injection of matriptase transfectants into athymic mice. Interestingly, further work showed that a secreted type of GlcNAcT-V protein promotes angiogenesis both *in vitro* and *in vivo* at physiological concentrations[82]. Although GlcNAcT-V is a Golgi enzyme, it is also secreted by cultured cells[72]. The authors proposed a new mechanism of GlcNAcT-V related tumor metastasis, which is not controlled by glycosylation. GlcNAcT-V secreted from cancer cells releases fibroblast growth factor (FGF-2) from heparan sulphate proteoglycan. The FGF-2 binds to FGF-2 receptor forming a ternary complex with associated heparan sulfate. It was also observed that a basic region on GlcNAcT-V was required for the release of FGF-2.

1.7 Inhibitors for GlcNAcT-V

GlcNAcT-V transfers an N-acetylglucosamine residue to glycopeptides bearing the minimum heptasaccharide sequence **1** (Fig. 15) converting it to the corresponding octasaccharide **2**[83]. Intensive studies have been done to probe the active site of this enzyme by making acceptors and inhibitors and measuring the degree of transfer or inhibition respectively. Initially tetrasaccharides containing part of the natural heptasaccharide were synthesized by Tahir *et al.*[84] (structure **3**) and Khan *et al.*[85]

(structure **4**). Tetrasaccharide **3** was a substrate for GlcNAcT-V, while tetrasaccharide **4** was very poorly recognized by the enzyme. Since Lemieux discovered that carbohydrates having a trisaccharide are frequently enough for faithful recognition by proteins[86] researchers started to examine trisaccharides as potential substrates for GlcNAcT-V.

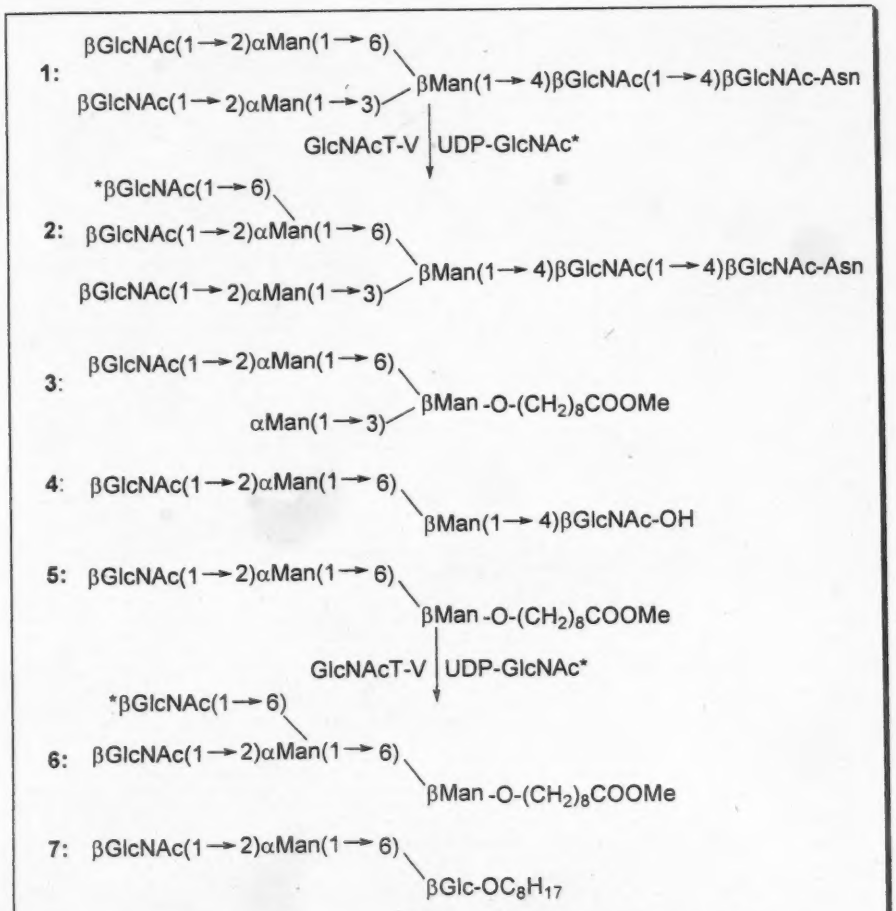


Fig. 15: Natural and synthetic substrates for GlcNAcT-V

1.7.1 Inhibitors with Modified β-Mannose/β-Glucose Residue

Tahir *et al.*[84] also synthesized a trisaccharide (structure **5**) which was assayed against GlcNAcT-V derived from human, mouse or hamster tissues[87]. It was indeed an

acceptor with the highest specific activity for the mouse lymphoma enzyme. Further work on Rous sarcoma virus-transformed baby hamster kidney cells showed that the enzyme effectively transfers a GlcNAc residue to this synthetic trisaccharide **5** converting it to the corresponding tetrasaccharide **6**[88]. The product was identified by comparison of its ^{1}HNMR spectrum to that of the authentic tetrasaccharide, which was prepared by chemical synthesis. It is interesting to note that the 8-methoxycarbonyloctyl aglycone was used to replace the chitobiose unit in the natural substrate because this hydrophobic group facilitates adsorption on reverse phase (C_{18}) chromatography supports. Later the trisaccharide **5** was tested against purified rat kidney cells and it had a K_m of 87 μM under the conditions used in the experiment[69]. In 1988, Srivastava *et al.*[89] synthesized an analog of compound **5**, trisaccharide **7**, which has a β glucose sugar instead of the β mannose and the octyl aglycone replacing the 8-methoxycarbonyloctyl moiety (Fig. 15). The compound was an excellent substrate with K_m 23 μM using purified hamster kidney cells as a source of the enzyme[90].

In order to investigate in more detail the molecular specificity of the enzyme, a series of analogs[89] was synthesized having the glucose moiety as the terminal sugar as it greatly simplifies the chemical synthesis of the acceptors compared to the original β mannose. Figure 16 shows the series of analogs synthesized to probe the glucose moiety. These data show that there is a low recognition of the reducing end sugar residue by the enzyme. Both the 4-deoxy (**8**) and 4-methoxy (**9**) glucose trisaccharides were found to be good acceptors. The two rotamer gg (**10**) and gt (**11**) about the C-5-C-6 bond in the 6-R methyl and 6-S methyl trisaccharide respectively were also prepared to investigate which of the two conformations was recognized preferentially by the enzyme. The gt conformer has the so-called ω angle (H-5-C-5-C-6-O-6) = -60° while the gg conformer has ω = 180°. The gt rotamer was found to be twice as active as parent **7** while the gg rotamer was only about half as active. However, because of the flexibility in these systems, this conformational analysis must be regarded as tentative. Finally the last analog involved

replacement of the glucose moiety by a cyclohexylmethyl ring (**12**). This compound had only 25% of the activity of **7**.

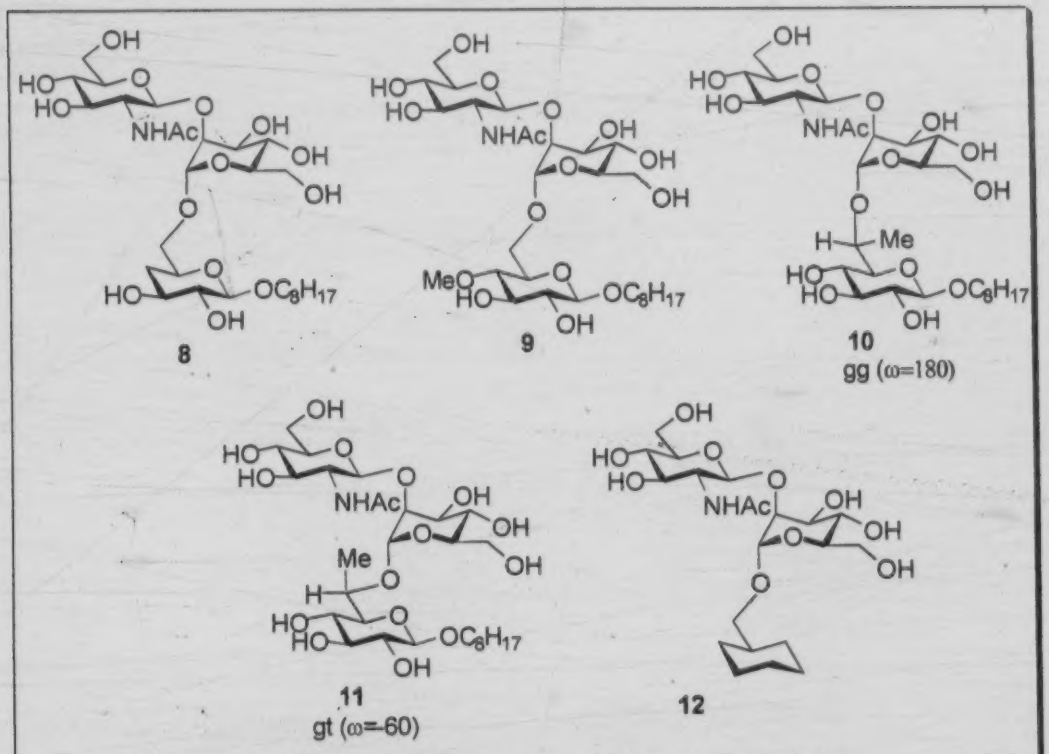


Fig. 16: Analogs synthesized to probe the glucose moiety

In 1989, Khan *et al.*[91] synthesized a series of trisaccharides β-D-GlcNAc-(1→2)-α-D-Man-(1→6)-β-D-Man-O-$C_6H_4NO_2$, β-D-GlcNAc-(1→2)α-D-Man-(1→6)-β-D-Glc-O-$C_6H_4NO_2$, β-D-GlcNAc-(1→2)-α-D-Man-(1→6)-β-D-Glc-O-Allyl and the tetrasaccharide β-D-GlcNAc-(1→2)-α-D-Man-(1→6)-β-D-Glc-(1→4)-β-D-Glc-O-Allyl as potential substrates for GlcNAcT-V. They preferred to use different aglycones from the octyl group because these nitro and allyl groups could be manipulated to prepare conjugates. One year later the same group synthesized the trisaccharide β-D-GlcNAc-(1→2)-4-O-methyl-α-D-Man-(1→6)-β-D-Glc-O-$C_6H_4NO_2$ as a potential acceptor-substrate[92].

Two conformationally restricted trisaccharide analogues were synthesized and evaluated as substrates for GlcNAcT-V[93]. Fig. 17 shows the structure of the two conformers. The possibility for rotation about the C-5-C-6 bond of Glc was eliminated by linking O4 and C6 with an ethylene bridge. It was found that the gg conformer (**13**) had a K_m of 29 μM while the gt conformer (**14**) gave a K_m of 9800 μM towards GlcNAcT-V from hamster kidney. These results indicate that GlcNAcT-V preferentially recognizes carbohydrate chains of N-glycoproteins in their gg conformation.

In order to extend earlier studies, Khan *et al.* synthesized the tetrasaccharide β-D-GlcNAc-(1→2)-α-D-Man-(1→6)-β-D-Glc-(1→4)-β-D-GlcNAc-OH which is quite similar to the natural heptasaccharide **1** as potential substrate for GlcNAcT-V[94]. However, it was poorly recognized by the enzyme.

Two trisaccharides, 2,3,4-O-trimethyl (**15**) and 2,3,4-O-tribenzyl (**16**) glucose analogs of **7** were prepared in order to determine whether the hydroxyl groups on the Glc residue are important for recognition[95]. Fig. 17 shows the structure of the trisaccharide analogs prepared to probe the glucose moiety. They were both excellent acceptors for GlcNAcT-V from hamster kidney cells with K_m of 33 μM (trimethyl analog) and 58 μM (tribenzyl analog). The disaccharide, β-D-GlcNAc-(1→2)-α-D-Man-O-C_8H_{17}, where the glucose moiety was removed, was found to be a poor substrate with a K_m of almost 2 mM.

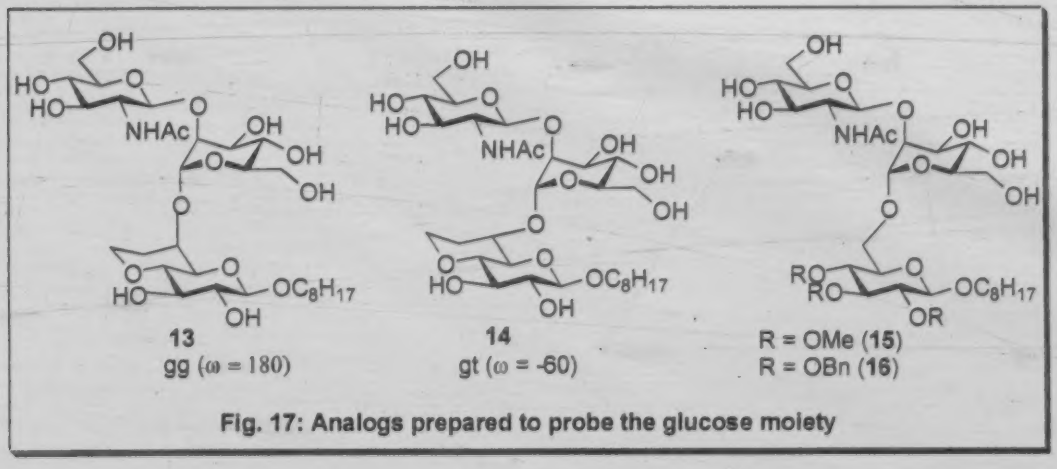


Fig. 17: Analogs prepared to probe the glucose moiety

Finally, a series of oligosaccharides were prepared by Brockhausen *et al.*[96] and evaluated for GlcNAcT-V activity from hamster kidney. All compounds with the minimum structure β-D-GlcNAc-(1→2)-α-D-Man-(1→6)-β-D-Glc/Man-R were good substrates for GlcNAcT-V. Furthermore, structures with biantennary structures were preferred over linear substrates. Fig. 18 shows a summary of the tolerance accepted at the glucose ring. After analysis of all these experiments, it can be concluded that the hydroxyl groups of the glucose terminal sugar do not play an important role during the enzyme-substrate complex formation. However removal of the glucose residue[89,95] is detrimental to enzyme activity suggesting that a specific hydrophobic recognition of glucose and its aglycon is required for activity.

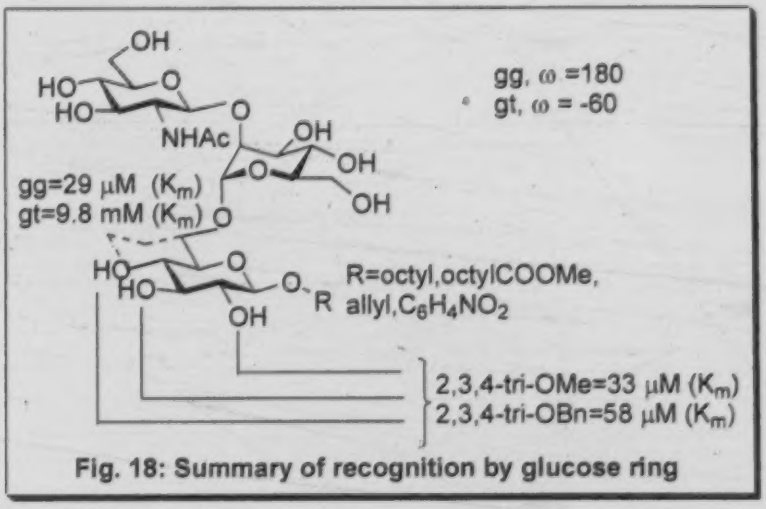


Fig. 18: Summary of recognition by glucose ring

1.7.2 Inhibitors with Modified α-Mannose Residue

Fig. 19 shows the structure of some of the analogs prepared to probe the mannose moiety. A deoxygenated analog of parent **7**, β-D-GlcNAc-(1→2)-6-deoxy-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} (**17**), was found to be a competitive inhibitor ($K_i \approx 70$ μM) for the enzyme from three sources: baby hamster kidney from parental cells, Rous sarcoma virus transformed cells and L-phytohemagglutinin-resistant cells[97]. Compounds made

previously[84,89] were also found to have similar K_m values for these three sources of enzyme. These data show that the active sites of GlcNAcT-V from these three sources are nearly identical. The fact that the 6' deoxy analog was an inhibitor shows that hydrogen bonding between the reactive hydroxyl group and a basic group on the protein is not essential for substrate recognition. Most probably the developing proton is transferred to a basic group only once glycosyl transfer is in progress or is directly transferred to the pyrophosphate or water molecules[98].

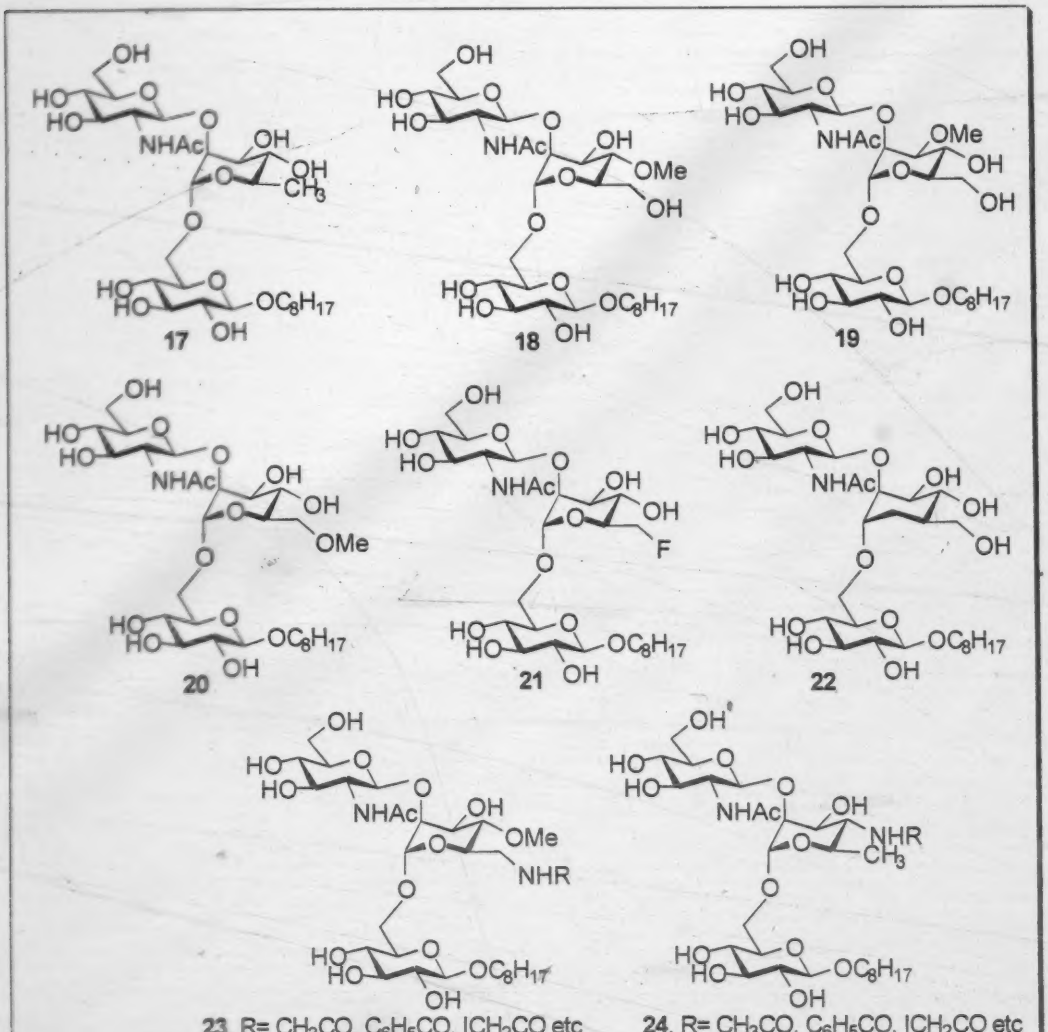


Fig. 19: Analogs prepared to probe the mannose moiety

Surprisingly, the trisaccharide β-D-GlcNAc-(1→2)-4-O-methyl-α-D-Man-(1→6)-β-D-Glc-OC_8H_{17} (**18**) was not a substrate for GlcNAcT-V, even though the reactive 6'-OH group was present, but was found to be a good competitive inhibitor with K_i=14 μM[90]. The 4' deoxy analog was synthesized in order to find out whether the 4'-hydroxy group was involved in recognition during reorganization of the active site toward the transition state for the transfer reaction. It was found to be a good substrate with a K_m of 74 μM. This clearly shows that the 4'-hydroxy group is not required for reaction. Therefore it can be concluded that the 4'-O-methyl analog binds to the enzyme but, being a bulkier group, it sterically preludes the transfer reaction.

In 1993, Khan *et al.*[99] synthesized two trisaccharides analogs, β-D-GlcNAc-(1→2)-6-O-methyl-α-D-Man-(1→6)-β-D-Glc-$C_6H_4NO_2$ and β-D-GlcNAc-(1→2)-4,6-di-O-methyl-α-D-Man-(1→6)-β-D-Glc-$C_6H_4NO_2$, as potential inhibitors for GlcNAcT-V. The 4',6'-di-O-methyl derivative was found to be an inhibitor of GlcNAcT-V from hamster kidney, hen oviduct microsomes and acute and chronic myeloid leukemia leukocytes[96].

One year later, three trisaccharides with modified mannose residues, β-D-GlcNAc-(1→2)-3-O-methyl-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} (**19**), β-D-GlcNAc-(1→2)-6-O-methyl-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} (**20**) and β-D-GlcNAc-(1→2)-6-fluoro-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} (**21**), were evaluated as potential acceptors and inhibitors for GlcNAcT-V using partially purified preparation from hamster kidney cells[100]. The 3'-O-methyl was an excellent substrate with a K_m of 60 μM. The 6'-O-methyl and 6'-fluoro do not possess the required 6-OH group and therefore cannot be substrates for the enzyme. However, they are both good inhibitors with K_i 70 (6'-O-methyl) and 24 (6'-fluoro).

A carbocyclic analog of **7**, β-D-GlcNAc-(1→2)-5a-carba-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} (**22**), where the ring oxygen of the mannose was replaced by a methylene group was synthesized and evaluated as a substrate for GlcNAcT-V from both purified

hamster kidney cells and cloned rat kidney[101]. It was indeed an acceptor for both sources of enzymes with $K_m \approx 28$ μM and 40 μM for the cloned GlcNAcT-V and purified GlcNAcT-V respectively. This work shows that the ring oxygen of the mannose ring is not involved in recognition by GlcNAcT-V.

A library of compounds was prepared by Lu *et al.*[102] and the key intermediate used was β-D-GlcNAc-(1→2)-6-amino-6-deoxy-4-O-methyl-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17}, where the 6'-hydroxy group has been replaced by an amino group and the 4'-hydroxy group with a methyl group (**23**). As noted earlier, the large methyl group at the 4' position is a good inhibitor though it sterically prevents the formation of products. Thus the introduction of this group would make sure that no transfer would occur which could potentially complicate interpretation of the results. The amino group was derivatized by either alkylation or acylation with a variety of compounds ranging from hydrophilic, hydrophobic, charged, aromatic and potential covalently inactivating groups. Surprisingly, all the compounds were competitive inhibitors with K_i values ranging from 21 to 297 μM and the strongest inhibitor was the N-iodoacyl derivative. The trend showed that anionic or hydrophobic groups were preferred near the site of transfer. Since substitutions at the 6'-hydroxy position seem to be tolerated by the enzyme, it can be concluded that during E-I complex, the reactive OH group does not make important contacts with the enzyme active site.

Another series of compounds[103] were made by the same authors where the key intermediate was β-D-GlcNAc-(1→2)-4-amino-4,6-dideoxy-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} (**24**) where the 4'-NH_2 was derivatized with a variety of compounds used previously. Once again, all the compounds were competitive inhibitors with K_i ranging from 3 to 106 μM when evaluated with GlcNAcT-V from two sources namely isolated hamster kidney and cloned rat kidney. This time the best inhibitor was with the hydrophobic C_6H_5CO- group. The two last sections clearly show that the C-4-C-6 end of the α-Man residue is not involved in recognition during the E-I complex formation.

A mixed library of 325 compounds using the key intermediate employed by Lu *et al.*[103] was prepared by solid phase combinational chemistry[104]. Frontal affinity chromatography coupled to electrospray mass spectrometry (FAC/MS) was used to screen and rank the compounds in order of highest protein binding affinity. This new technique provides a faster analysis of a large library of compounds and easily identifies the tightest inhibitors. Fig. 20 outlines the substitutions tolerated at the mannose ring. It can be concluded that none of the hydroxyl groups on the mannose of 7 contribute importantly to recognition by the enzyme.

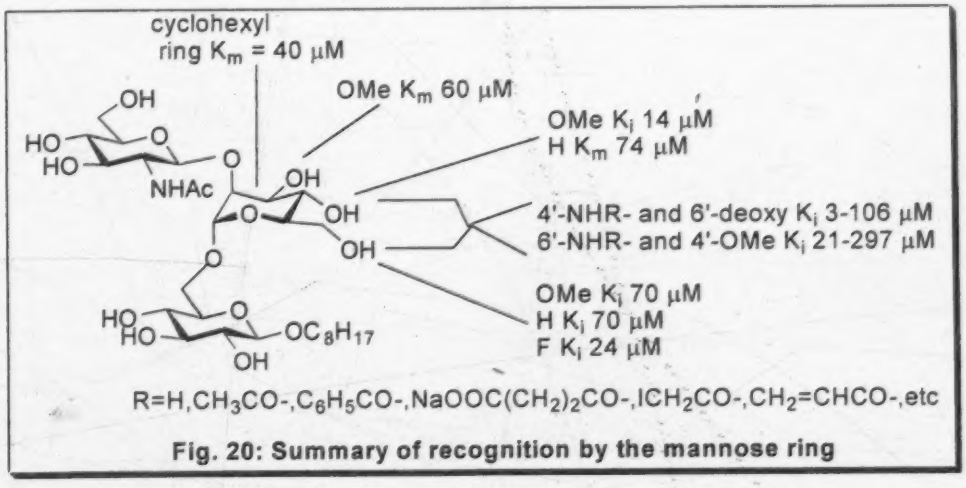


Fig. 20: Summary of recognition by the mannose ring

1.7.3 Inhibitors with Modified N-Acetylglucosamine Residue

Fig. 21 shows the structure of some of the trisaccharide analogs synthesized to probe the GlcNAc residue. Modification at the N-acetylglucosamine ring was carried out by Kanie *et al.* where the 4''-hydroxy group was replaced by OMe, F, NH_2, H, NHAc and inverted to the galacto configuration to give a series of compounds (**25a-f**)[105]. This work was inspired by the fact that when galactose is transferred to the 4'' position of the trisaccharide **7**, it is no longer a substrate for GlcNAcT-V. When the compounds were tested as acceptors or inhibitors towards GlcNAcT-V from hamster kidney cells, their

activity was very low giving a K_m in the range of 1.4 to 13 mM. Therefore it can be concluded that acceptor activity after galactosylation is impaired because it destroys important hydrogen bonding interaction with the enzyme active site and not by introducing steric bulk. The 4''-hydroxy group can be described as a key polar element for GlcNAcT-V.

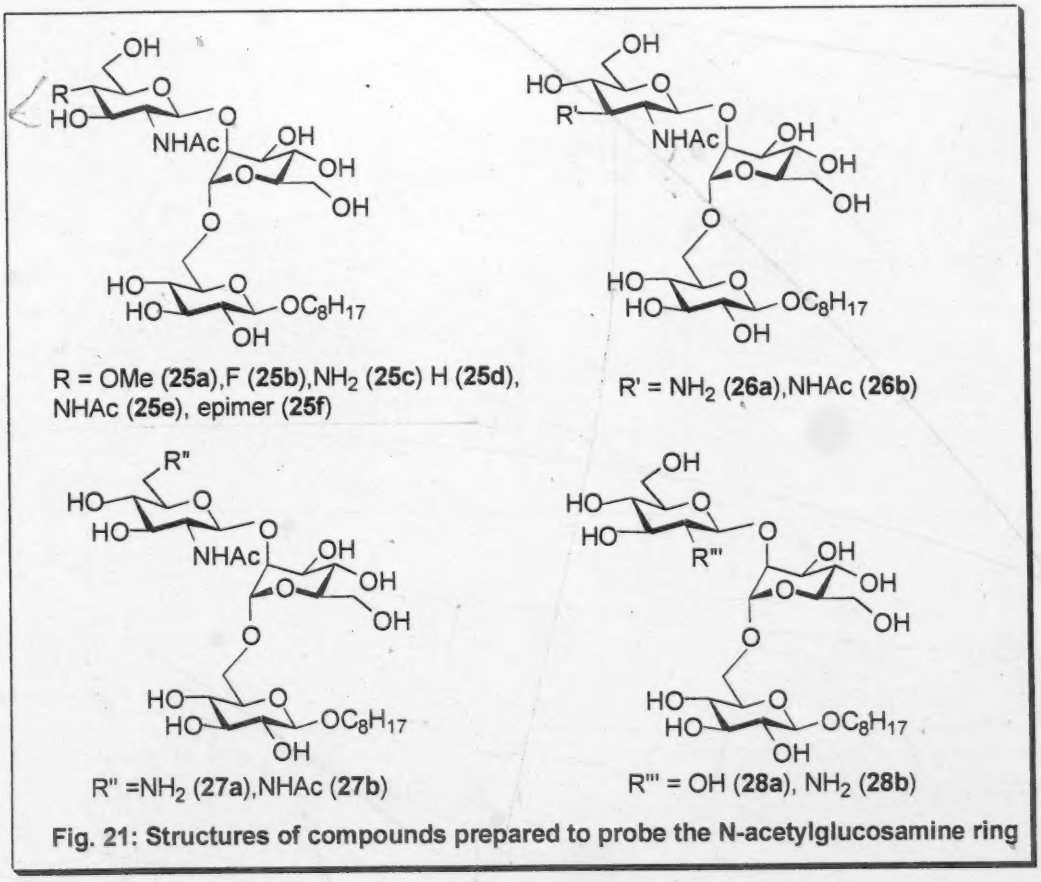


Fig. 21: Structures of compounds prepared to probe the N-acetylglucosamine ring

Four compounds were prepared where the 3''-hydroxy (compounds **26a** and **26b**) and 6''-hydroxy group (compounds **27a** and **27b**) of the GlcNAc ring was replaced, independently, by NH_2 and NHAc[106] (Fig. 21). When they were evaluated as substrates for GlcNAcT-V from hamster kidney cells, the 6'' analogs were found to be inactive up

to 10 mM while the 3'' analogs were very poor substrates with K_m of 1.1 and 1.9 mM. Therefore it can be concluded that all three OH groups on the GlcNAc residue are key polar groups which are required for enzyme activity.

In 1997, an analog of **7** was prepared where a Glc ring replaced the terminal GlcNAc sugar (**28a**). It was found to be a substrate for GlcNAcT-V from cloned rat kidney cells with a K_m of 111 μM[107]. The 2''-NH_2 analog of **7** (**28b**) was also prepared and it had a K_m of 160 μM[108]. This shows that the strategy for the design of inhibitors for GlcNAcT-V will be towards the generation of carbohydrate mimics where only the essential three OH groups are present. A summary of the recognition features exhibited by the GlcNAc ring is presented in Fig. 22.

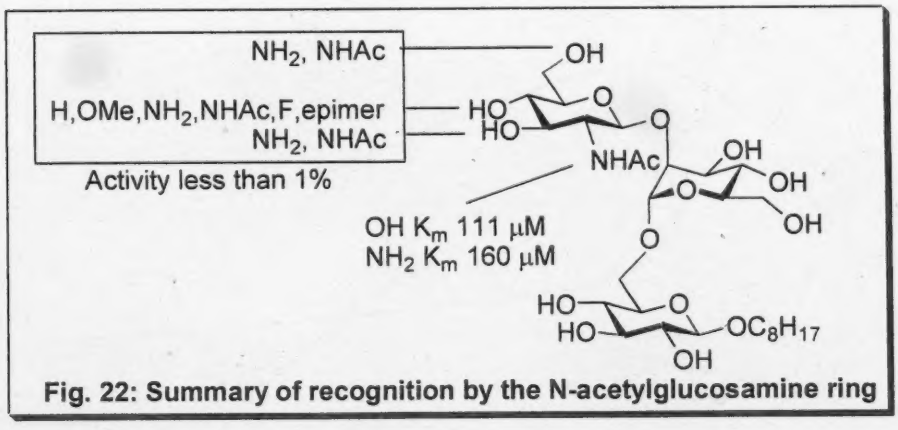


Fig. 22: Summary of recognition by the N-acetylglucosamine ring

1.7.4 Inhibitors with Thio Linkages

Two thio analogs of β-D-GlcNAc-(1→2)-α-D-Man-(1→6)-β-D-Glc-O-C_8H_{17} where the glycosidic linkages between the sugars were independently replaced with sulfur were synthesized. Both were found to be acceptors with K_m in the range of 300 μM. This shows that the enzyme tolerates substitution at the oxygen linkage of the acceptor[107].

1.7.5 Summary

Fig. 23 shows a summary of acceptor recognition by GlcNAcT-V

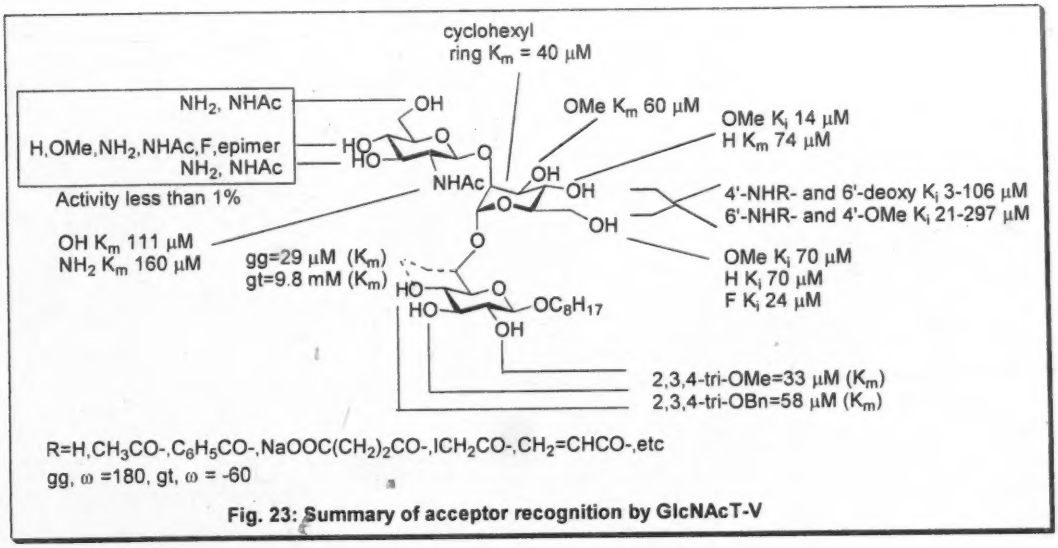


Fig. 23: Summary of acceptor recognition by GlcNAcT-V

1.8 Conclusion

Evidence shows that GlcNAcT-V is indeed an important enzyme in tumor progression and metastasis[13-18]. Therefore inhibiting the action of GlcNAcT-V could provide a new strategy for cancer therapy. Several approaches have been used and they are as follows:

1. Synthesis of inhibitors for GlcNAcT-V[84-107].
2. Suppression of ets-1, which is one of the most important transcriptional factors in up-regulation of GlcNAcT-V[76].
3. Understanding the mechanism of secretion of GlcNAcT-V in cancer cells and inhibiting its secretion.
4. Use of some acidic reagents to mask the basic domain required for GlcNAcT-V activity[82].
5. Degradation of GlcNAcT-V by proteolysis.

It is interesting to note that the concentration of the donor, UDP-GlcNAc can also be a critical factor in the formation of the β-1,6 branched glycoproteins. The donor has a relatively higher K_m value compared to other GlcNAc transferases suggesting that the action of GlcNAcT-V could be controlled *in vivo* by its concentration[109].

1.9 Scope of this Project

Since no crystal structure is available for GlcNAcT-V yet, researchers have been trying to probe the active site of this enzyme by synthesizing a variety of acceptors/inhibitors and measuring their activity towards this enzyme. This thesis research has the objective of synthesizing such inhibitors, which will bring some light in understanding the acceptor specificity of this metastasis-related enzyme. The design, synthesis and enzymatic evaluation of the new inhibitors are described in the next section.

Chapter 2

New Active-site Probes for GlcNAcT-V

2.1 Introduction

From the literature survey presented in Chapter 1, it can be seen that the synthesis and evaluation of inhibitors is useful in probing the active site of GlcNAcT-V. Therefore, it remains interesting to design, synthesise and evaluate new inhibitors for this enzyme. Useful and unexpected information may be obtained from such studies. This Chapter deals with the design and synthesis of the new active site probes for GlcNAcT-V while the following Chapter (Chapter 3) discusses the enzymatic evaluation of the new compounds towards GlcNAcT-V. Fig. 24 shows a schematic representation of the original acceptors (**7, 28a)** and the compounds synthesized.

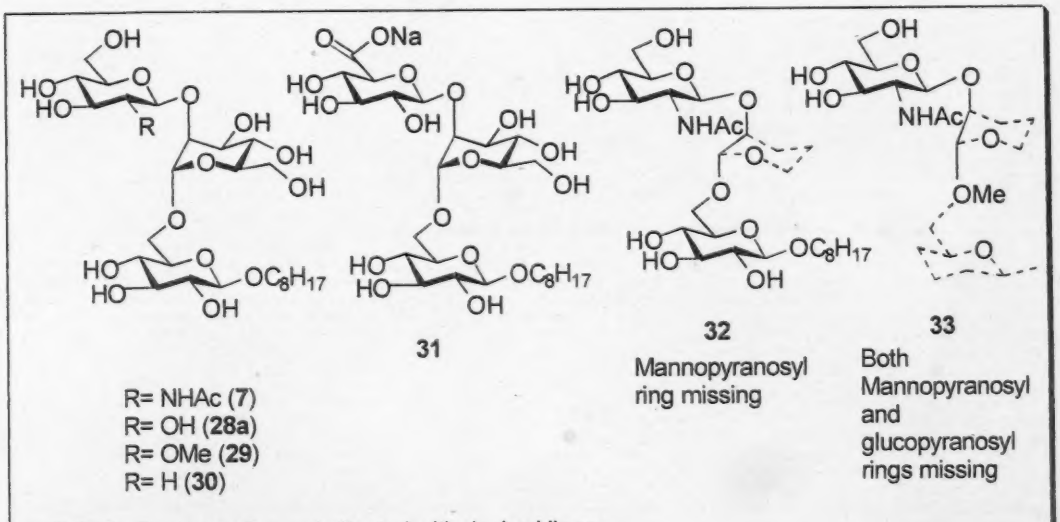


Fig. 24: Schematic representation of original acceptors (7, 28a) and compounds synthesized

2.2 Modification on the N-Acetylglucosamine Ring.

Various modifications could be envisaged when looking at the synthetic trisaccharide **7**, which was found to be an excellent substrate for GlcNAcT-V[89]. The first section deals with the modification on the N-acetylglucosamine ring.

2.2.1 Design and Synthesis of Octyl 2-O-methyl-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside (29)

The trisaccharide analog of **7** with a 2''-OH group instead of a 2''-NHAc group was previously shown to be a substrate with a K_m of 111 μM[107]. Both the OH group and the NHAc group retain the ability to be hydrogen-bond donors. The OH group is also much smaller than the NHAc group so it is not clear whether it can simply fit into the NHAc space or act as an H-bond donor. Therefore, it seemed interesting to synthesize the 2''-OMe analog of trisaccharide **7**, where H-bond donation is no longer possible from O-2. This group will also test the ability of the enzyme to tolerate a very large substitution at this site.

The retrosynthetic analysis leading to the trisaccharide **29** suggested the coupling of the three building blocks **34**, **35** and **36** as shown in Fig. 25.

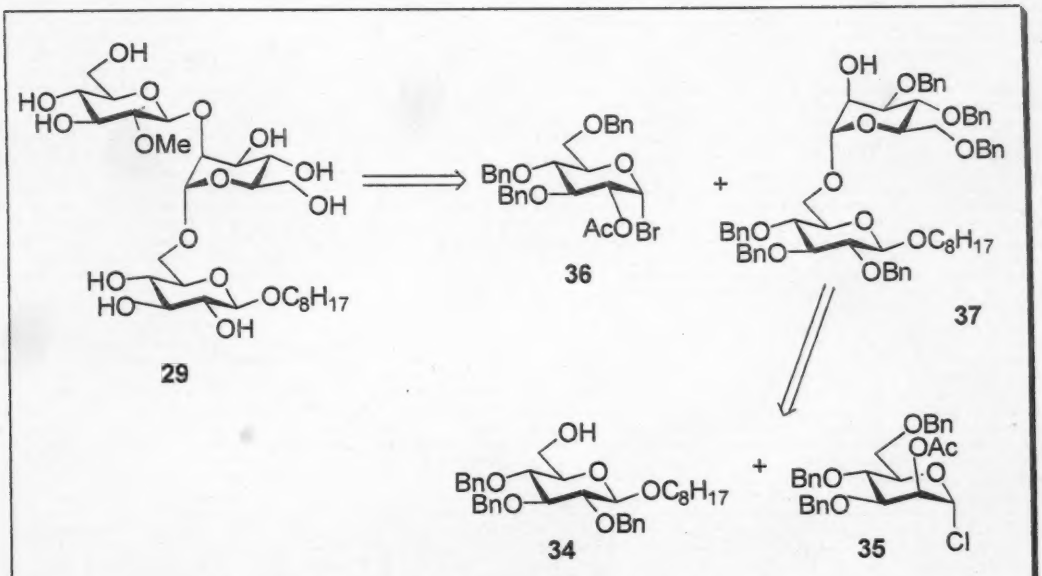


Fig. 25: Retrosynthetic analysis of trisaccharide 29

Compound **34** was obtained from the commercially available starting material penta-O-acetyl-β-D-glucopyranose **38** as shown in Fig. 26. Reaction with 33% hydrogen bromide in acetic acid gave the α-bromide, which reacted with octanol in the presence of silver trifluoromethanesulfonate and tetramethylurea to give the octyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside **39** in 60% yield. Zemplen deacetylation provided the deprotected sugar **40** in quantitative yield. Compound **34** was obtained from **40** using established procedures[89]. Selective protection of the primary alcohol was achieved by treatment with chloro p-methoxyphenyldiphenylmethane in pyridine to give **41**. Benzylation of the secondary alcohols with benzyl bromide and sodium hydride afforded **42**, which was treated with 80% acetic acid at room temperature to give the first building block **34** in 50% yield over the last three steps.

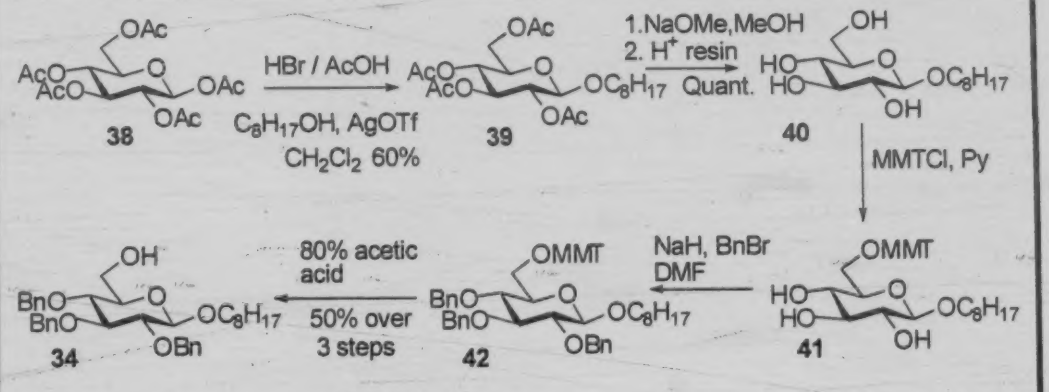


Fig. 26: Synthesis of building block 34

Building block **35** was obtained from D-mannose as starting material as shown in Fig. 27. D-mannose was acetylated using acetic anhydride in pyridine to quantitatively give the two anomers of the penta-O-acetyl-D-mannopyranose **43**. The α-bromide **44** was obtained by treatment with 33% hydrogen bromide in acetic acid. The 1,2-methyl orthoacetate **45** was formed upon treatment of bromide **44** with 2,6-lutidine and methanol[110]. Careful O-deacetylation of **45** with sodium methoxide and methanol provided the three alcohols that were immediately O-benzylated with sodium hydride and benzyl bromide making sure that the solution was always basic. The benzylated 1,2-methyl orthoacetate **46** was converted into the building block **35** by treatment with trimethylsilylchloride in high yield[111].

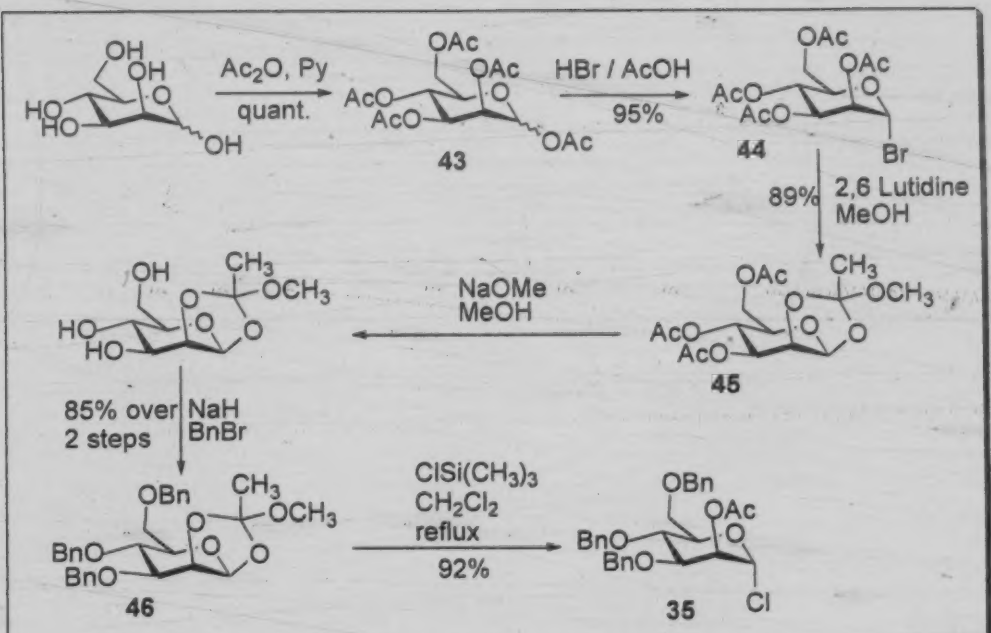


Fig. 27: Preparation of building block 35

Building blocks **34** and **35** were coupled (Fig. 28) in the presence of silver trifluoromethanesulfonate and tetramethylurea to furnish the disaccharide **47** in 85% yield[89]. The H-1' and C-1' at the new linkage were found to be at 4.90 ppm in the ^{1}H NMR and 97.9 ppm in the ^{13}C NMR spectra respectively. Usually a chemical shift greater than 4.75 ppm in the ^{1}H NMR and less than 100 ppm in the ^{13}C NMR would indicate that an α linkage has been formed. However a more definite proof would be to measure the coupling constant between the anomeric carbon and hydrogen. During a study performed on a number of hexopyranoses, it was observed that the direct coupling constants between the anomeric carbon atoms and protons, $J_{C\text{-}1,H\text{-}1}$, were found to be about 160 in the β-anomers and about 170 Hz in the α-anomers[112]. Since then, this useful NMR relationship (α linkages above 165 ppm and β linkages below 165 ppm) has been used to prove the kind of linkages generated after a reaction. In this case, a coupling constant of 172.9 Hz was obtained showing that indeed the new linkage was an

α linkage. Deacetylation of the 2'-OAc group in **47** afforded the disaccharide alcohol **37**, which was ready to act as an acceptor.

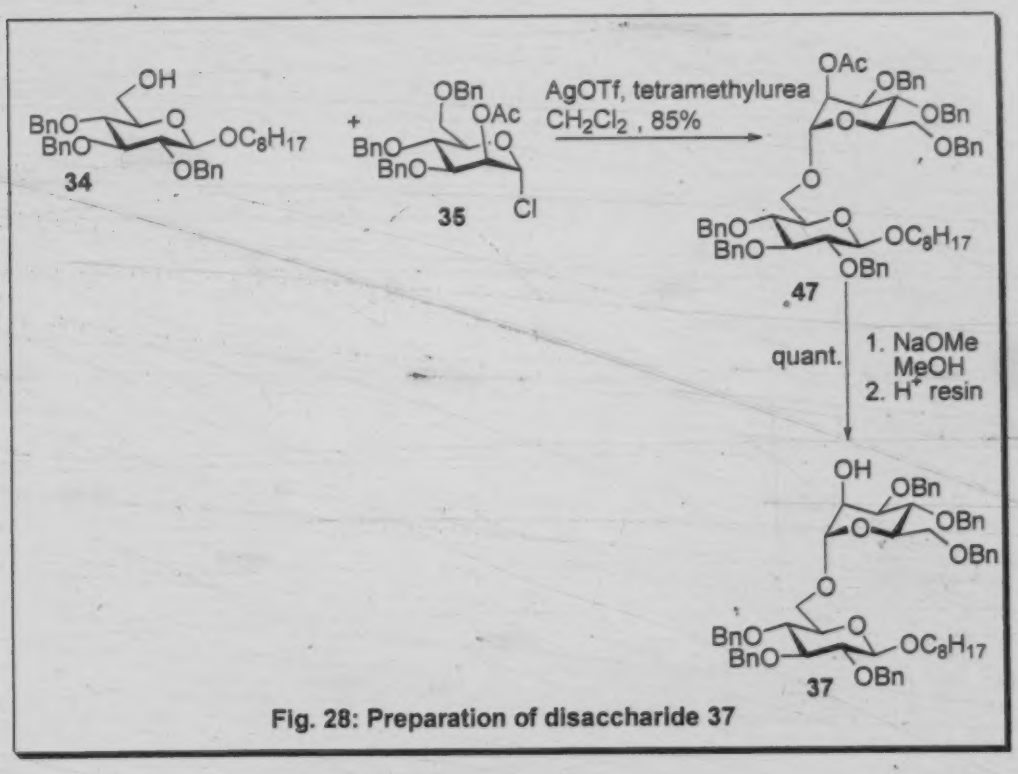


Fig. 28: Preparation of disaccharide 37

Finally donor **36** was obtained from the pentaacetate of glucose using procedures analogous to those shown in Fig. 27. The α-bromide **48** was produced upon treatment with 33 % hydrogen bromide in acetic acid (Fig. 29). This bromide was equilibrated with its β–bromide by treatment with Bu_4NBr and it was finally converted to the β-1,2 ethyl orthoacetate of glucose **49** as a mixture of the two diastereomers[113]. Careful O-deacetylation and subsequent O-benzylation was achieved, using the same procedure as in the preparation of **35**, to form **50**. Donor **36** was obtained as the main product (thermodynamic product) when **50** was treated with acetyl bromide in the presence of tetraethylammonium bromide[114].

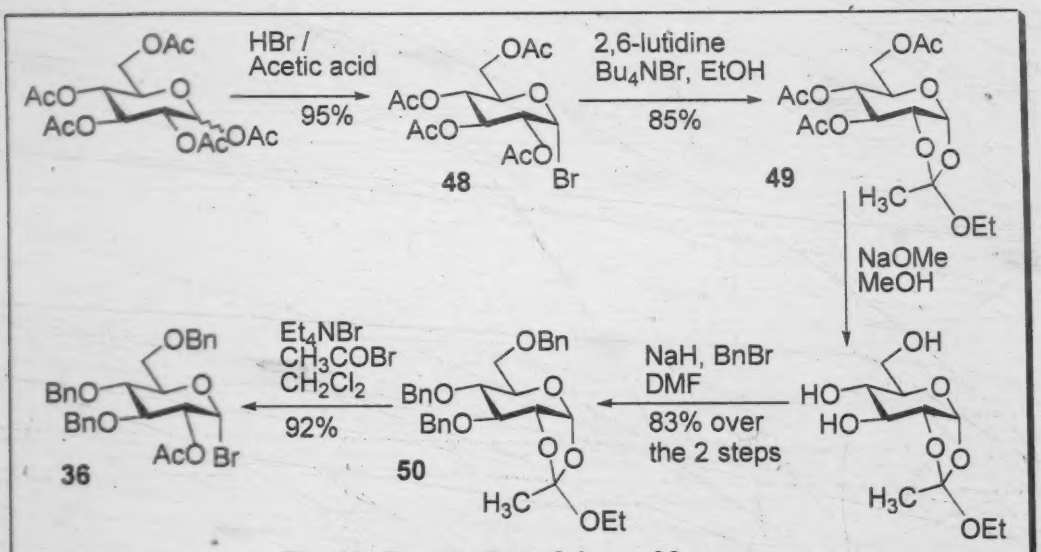


Fig. 29: Preparation of donor 36

Compound **51** was obtained in good yield by coupling donor **36** to the disaccharide **37** in the presence of silver trifluoromethanesulfonate and collidine as coupling reagents (Fig. 30)[115]. As noted earlier, the coupling constants of 157.9, 158.4 and 169.3 Hz between the anomeric carbons and protons clearly show that the compound has the required stereochemistry of two β and one α linkages. Zemplen deacetylation of the 2''-OAc provided the free alcohol **52**, which upon treatment with methyl iodide in the presence of sodium hydride gave the desired protected trisaccharide **53**. Finally debenzylation of **53** using hydrogen gas in the presence of catalytic 20% palladium hydroxide on carbon afforded the final trisaccharide **29** in 14 % overall yield from penta-O-acetyl-β-D-glucopyranose. Compound **29** was fully characterized by ^{1}H NMR, ^{13}C APT and high resolution ESMS. The coupling constants between the anomeric carbons and protons (160.8, 162.1 and 170.9 Hz) show that the desired stereochemistry at each linkages were present. The NMR data are collected in Table 1 and 2, Chapter 2, where they will be further discussed.

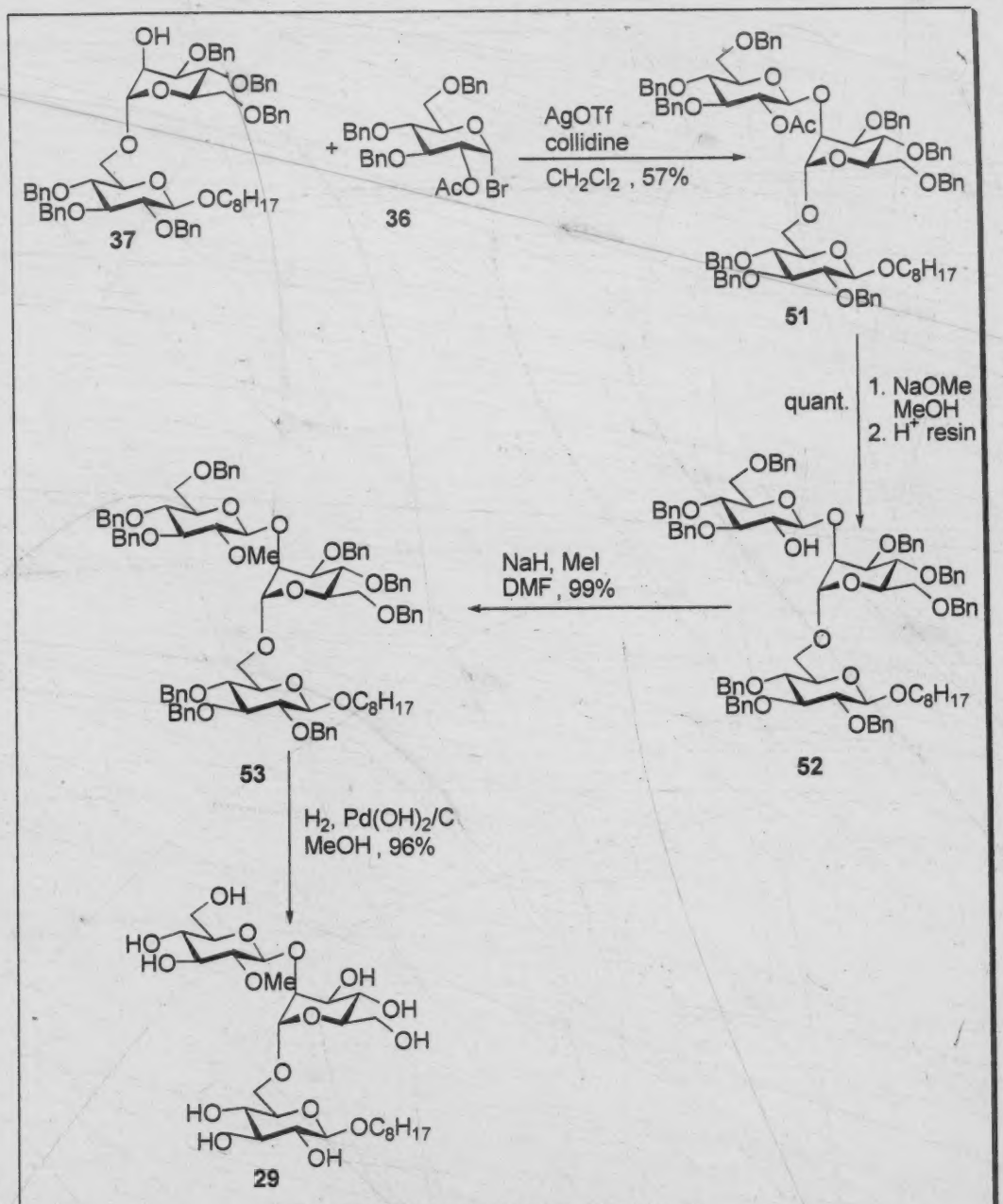


Fig. 30: Synthesis of trisaccharide 29

2.2.2 Design and Attempted Synthesis of Octyl 2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside (30)

In order to further explore the active site of the enzyme around the 2-position of the N-acetylglucosamine ring, the synthesis of another analog of the previously prepared **29** was attempted. It was thought that the deoxy analog **30** would be able to provide information about whether hydrogen bonding is required in that area. Therefore, the most obvious route was to deoxygenate at the 2'' position of compound **52** by a Barton-McCombie deoxygenation method as shown in Fig. 31.

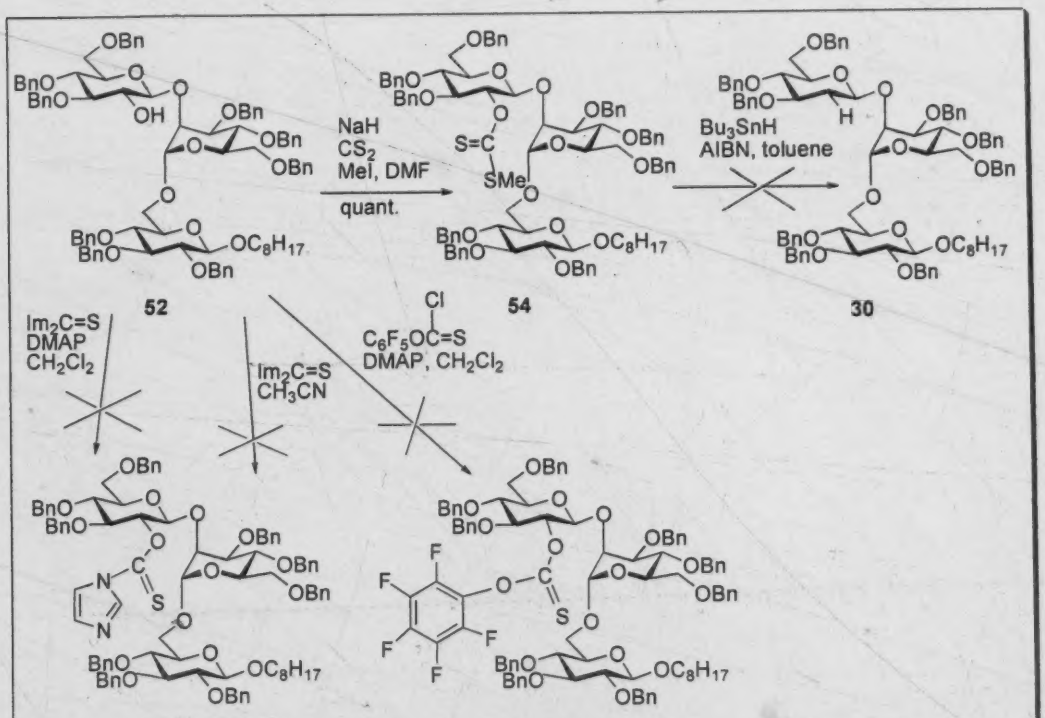


Fig. 31: Attempted syntheses of 30

Thus compound **52** was treated with sodium hydride, carbon disulfide and methyl iodide to form the methyl xanthate ester **54**. However upon treatment with tributyltin hydride in the presence of a catalytic amount of AIBN, a complex mixture was formed.

The separation of this mixture proved very difficult by column chromatography. The main product was identified as the 2''-hydroxy compound **52** while the minor products were decomposition products. Apparently, it seems that radical cleavage to the alcohol is much faster than deoxygenation[116]. The reaction was repeated several times but the same result was obtained. Therefore, in order to test the reagents and procedure used in this reaction, a simpler monosaccharide, octyl 4,6-benzylidene-2-deoxy-2-phthalimido-3-O-[(methylthio)thiocarbonyl]-β-D-glucopyranoside, was used as a starting material. However, in this case the deoxy product was formed in high yield (70%) showing that the reagents and technique were good. Fig. 32 shows the mechanism whereby the hydroxyl group may be formed instead of the desired deoxy compound **30**[117]. In this mechanism, addition of the tributylstannyl radical to sulphur is not followed by C–O fragmentation but by hydrogen capture and further reaction to give the alcohol. Moreover, it is useful to note that 2-deoxy compounds are usually very unstable so that even though it could be formed during the reaction it might be easily decomposed to side products.

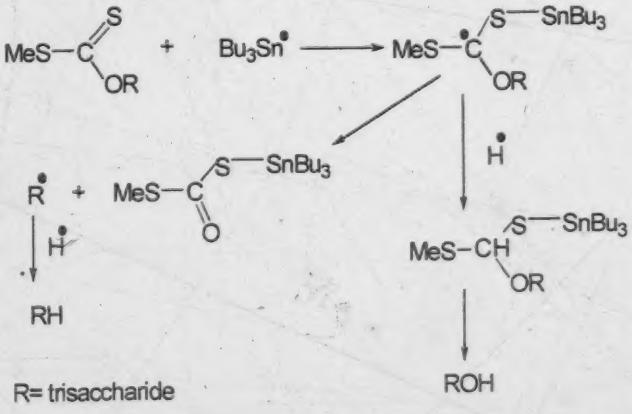


Fig. 32: Mechanism showing the formation of the alcohol during the deoxygenation reaction[116]

Having failed the deoxygenation using the methyl xanthate ester, it was hoped that the thiocarbonyl imidazolide derivative would work. Therefore, **52** was refluxed with thiocarbonyldiimidazole and DMAP in dichloromethane (Fig. 31). However, no product was formed after refluxing for two days. The usual protocol was slightly adapted and acetonitrile was used instead of dichloromethane as solvent[118]. However, again no detectable amount of product was formed. At this stage another commonly used thiocarbonyl reagent (pentafluorophenyl chlorothionoformate) was employed. Unfortunately, no product was obtained once again. Fig. 31 shows the various attempted routes, which failed during the synthesis of trisaccharide **30**. Most probably the trisaccharide **52** is oriented in such a way that it cannot afford to have bulky substituents at the 2'' position during the thionoacylation which would produce a sterically demanding sp^3-hybridized intermediate.

This was indeed rather discouraging because the deoxygenation cannot be made on the donor **36** prior to coupling with the disaccharide **37**. This is because the group at the 2'' position (OAc) is a participating group, which directs the coupling to give the desired β-linkage. Displacement reactions were not attempted as these are usually sluggish on carbohydrate compounds and furthermore the instability of the 2''-deoxy trisaccharide **30** might even make it difficult to isolate it in high purity. Therefore, the synthesis of trisaccharide **30** was abandoned.

2.2.3 Design and Synthesis of Octyl β-D-glucopyranosyluronic acid-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside sodium salt (31)

It is known that the 3, 4 and 6 hydroxyl groups on the terminal N-acetylglucosamine ring of the trisaccharide **7** are key polar elements for recognition during the E-S/E-I complex formation[108]. Therefore, these groups should be incorporated if a successful substrate/inhibitor is to be designed. However, a slight modification at the 6'' position whereby a carboxyl group replaces the hydroxyl group (trisaccharide **31**) was

envisioned as potentially yielding a compound that might bind should the enzyme be an H-bond donor to OH-6, or use an ammonium base for the interaction. There are three main differences between the hydroxyl and the carboxyl groups. Firstly, the hydroxymethyl group has a hydrogen atom which is absent in the sodium salt of the carboxyl trisaccharide. Secondly, the carboxyl group is charged, while the hydroxyl group is neutral. Finally, the carboxyl group has an additional carbonyl group. This means that if the trisaccharide acts as a hydrogen donor during hydrogen bonding interaction, then definitely trisaccharide **31** would be impaired as a substrate/inhibitor because this important recognition bonding would not be possible. However if the trisaccharide acts as a hydrogen acceptor, then trisaccharide **31** would be an almost equivalent substrate/inhibitor or even a better one due to the presence of the carbonyl oxygen. Another conclusion can be made about the types of amino acids recognised by the 6''-OH from the fact that the negatively charged trisaccharide **31** would act as a good substrate if the amino acids are positively charged while negatively charged amino acids (e.g. Asp, Glu) would greatly affect binding. A schematic representation of these interactions by both the hydroxyl and carboxyl compounds are shown in Fig. 33. These two observations can work together to reinforce a particular conclusion. Therefore, the synthesis of **31** was embarked upon with the hope that it would provide useful information.

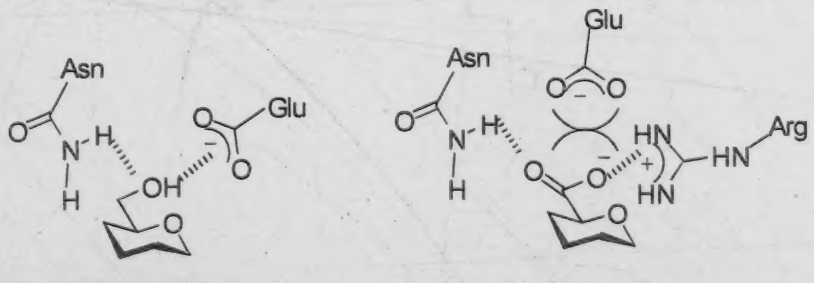


Fig. 33: Interactions favored/disfavored by the hydroxyl and carboxyl groups

Various synthetic strategies could be employed for the preparation of trisaccharide **31** where one could possibly envisage starting with the methyl β-D-glucopyranoside and introducing appropriate protecting groups, oxidation of the C-6 and finally acetolysis of the methyl glycoside. However, many authors have observed that this acetolysis does not work well on a large scale[119]. As a result it was thought wise to employ the D-glucofuranurono-3,6-lactone **55** as starting material that would avoid the oxidation step, which can sometimes be troublesome.

The initial attempt to prepare **31** involved the building blocks **56** and disaccharide **37** as shown in the retrosynthetic analysis of **31** (Fig. 34). Therefore, the synthesis of donor **56** began with the commercially available D-glucofuranurono-3,6-lactone **55**, which was converted into the methyl glucurono ester **57** in the presence of methanol and catalytic sodium hydroxide. Acetylation of **57** using acetic anhydride and pyridine provided the methyl tetra-O-acetyl-D-glucopyranuronate **58**. Finally, reaction with 33% hydrogen bromide in acetic acid furnished the α-bromide **56**[120] as shown in Fig. 35.

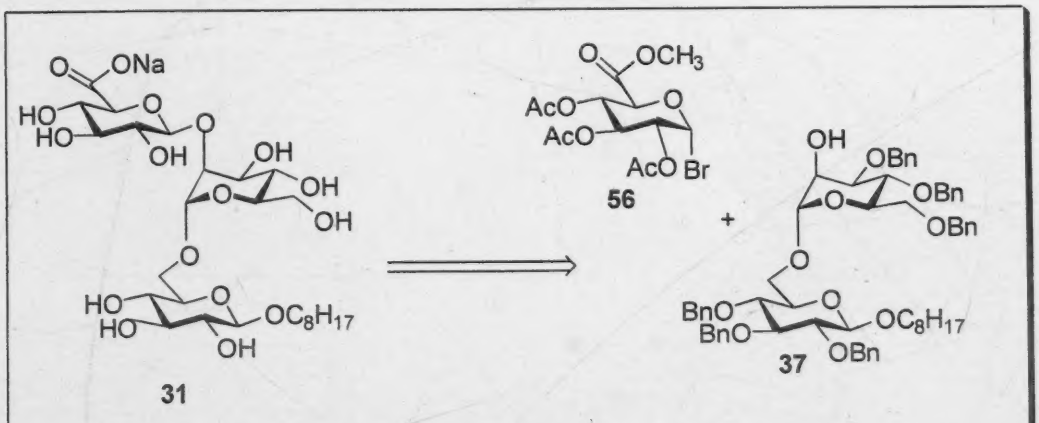


Fig. 34: Retrosynthetic analysis of 31

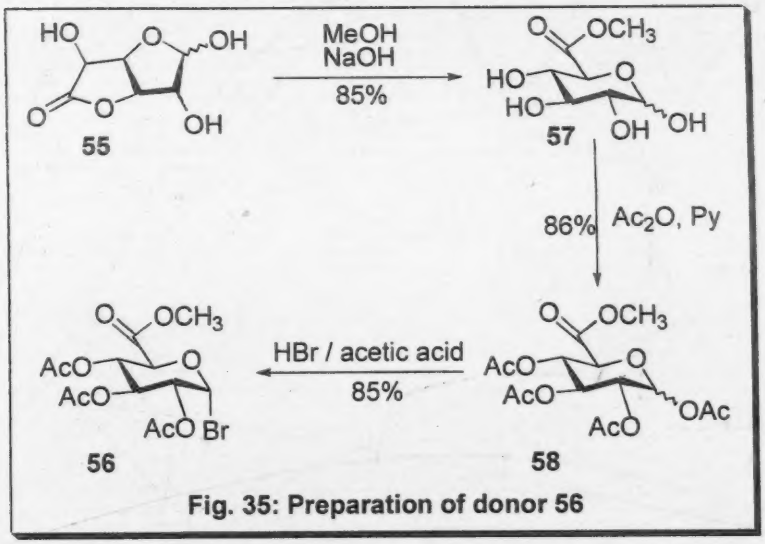


Fig. 35: Preparation of donor 56

Attempted coupling of the bromide **56** with the disaccharide **37**, in the presence of silver trifluoromethanesulfonate[121], proved unrewarding as a complex mixture was obtained (Fig 36). The reaction was repeated several times but the same result was obtained. The reaction was modified through inclusion of tetramethylurea in the preparation[122]. However, the same result was obtained. Attempts to separate this mixture were not successful. Donor **56** has previously been reported as sometimes giving low or no yield of product[123]. This was attributed to the fact that the 2'-OH in mannose is a relatively less reactive position so that a reactive donor is required. Furthermore, the electron withdrawing methyl ester group makes it even more difficult to form the carbocation at the anomeric position.

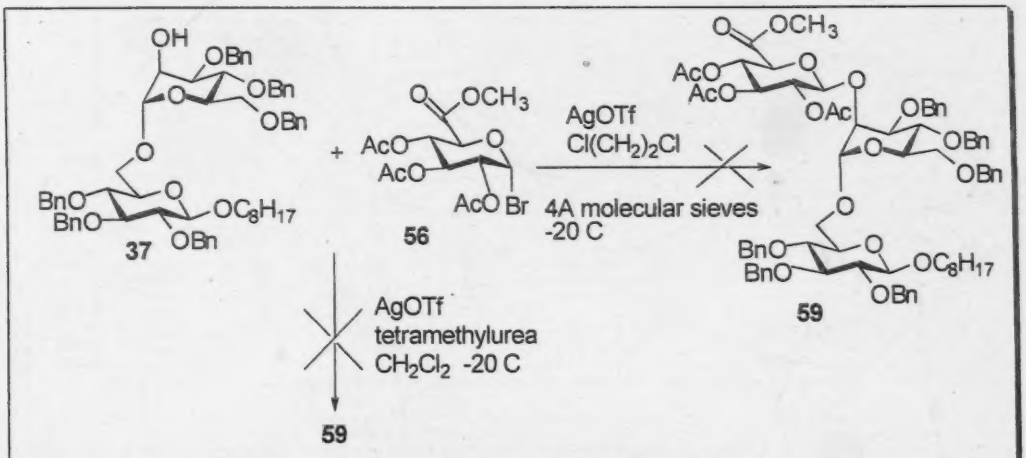


Fig. 36: Failed synthesis of trisaccharide 59

To circumvent this problem, we decided to use a benzoylated donor **60**. The synthesis of **60**[124] started with **57** and is shown in Fig. 37. Compound **57** was benzoylated with benzoyl chloride and pyridine to form **61** in 90% yield. It was preferred to prepare the α-trichloroacetamidoyl derivative rather than the bromide. Therefore, selective debenzoylation at the anomeric position was achieved by using hydrazine acetate on **61** and carefully monitoring the reaction to give the corresponding hemiacetal (**62**) in 66% yield. A mixture of the hemiacetal was reacted with trichloroacetonitrile and DBU until it was fully converted into the thermodynamic product α-trichloroacetamidate **60** (53.5 % overall yield).

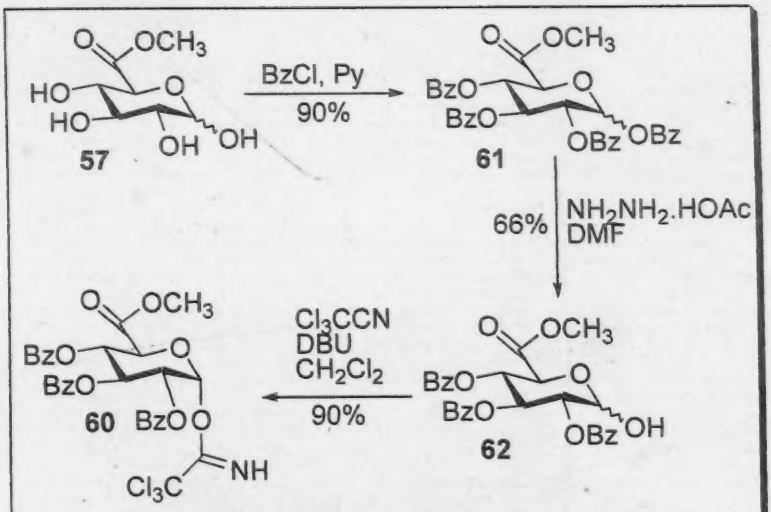


Fig. 37: Preparation of donor 60

Having obtained the donor **60**, we proceeded to couple it to the disaccharide **37** in the presence of trimethylsilyl triflate as the promoter (Fig. 38). The reaction proceeded smoothly to give exclusively the trisaccharide **63** in 91% yield. The anomeric carbon of this new linkage was found at 99.2 ppm in the ^{13}C NMR spectrum and the coupling constant of 162.2 Hz between the anomeric carbon and proton in this trisaccharide **63** effectively shows that the linkage between the glucuronate ester and the mannose ring has the β configuration. Hydrogenolysis of **63** using 20% $Pd(OH)_2/C$ in methanol gave **64**. Subsequent deacetylation of **64** using sodium hydroxide in a mixture of methanol and water at 0° C afforded the desired trisaccharide **31**. However, the final deprotection had to be carefully done so as to reduce the elimination side product (the 4,5-ene). The product can easily be separated from this side product by passing it through a Sephadex G-10 column and eluting with water. The anomeric protons were found at 5.01 (1.3 Hz), 4.54 (8 Hz) and 4.45 (8 Hz) in the ^{1}H NMR spectrum. Furthermore, chemical shifts of 103.1. 102.3 and 98.4 ppm with coupling constants of 161.2, 162.2 and 171.5 Hz respectively between the anomeric carbons and protons clearly show that the desired

linkages (two β and one α) are present on the deprotected trisaccharide **31**.

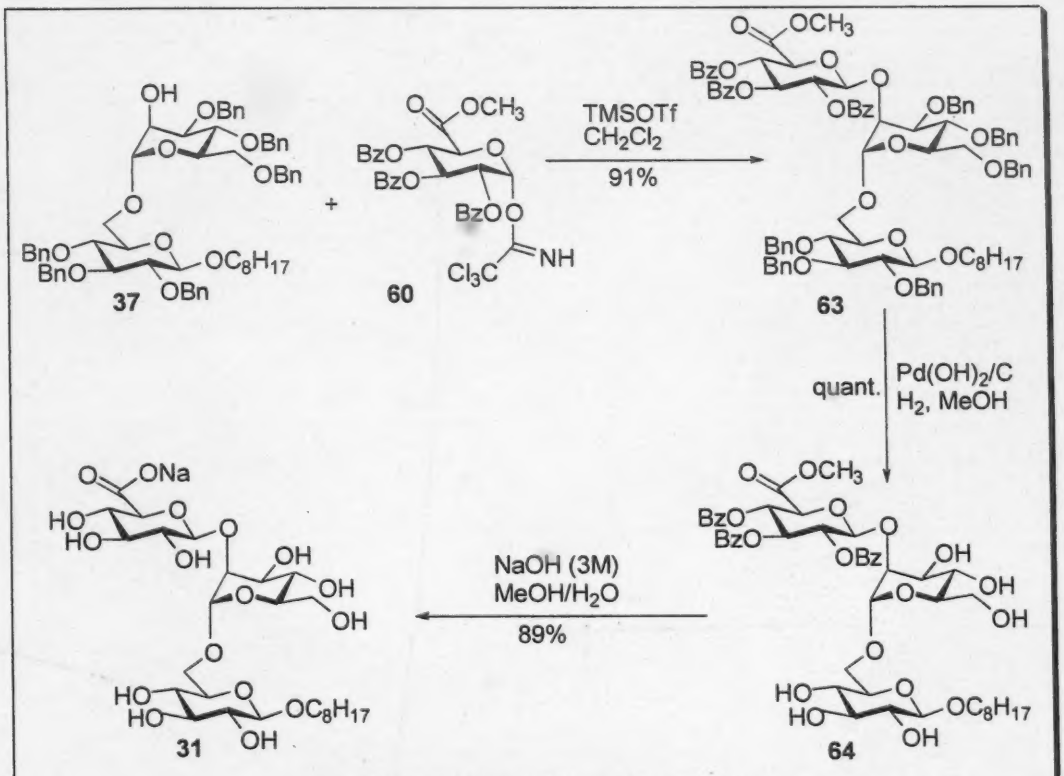


Fig. 38: Synthesis of trisaccharide 31

2.3 Simplification of the Mannose Ring

This section deals with the synthesis of compounds with a simplified mannose ring.

2.3.1 Design and Synthesis of Octyl 6-O-[(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-hydroxyethyl]-β-D-glucopyranoside (32)

From the literature survey presented in Chapter 1, it was found that the mannose ring tolerates broad substitutions at all of its OH-groups[90,96-104]. Furthermore, the ring oxygen was found not to be involved in recognition as it can be replaced by a methylene group and still act as an acceptor[101]. Therefore we thought that it would be interesting to

prepare a disaccharide **32**, whereby a hydroxyethyl group replaces the mannose ring and determine whether it can still act as an inhibitor for the enzyme. While such a compound would certainly be more flexible, it might still retain measurable inhibitor activity.

The synthetic strategy for the synthesis of the disaccharide **32** involved three building blocks, namely **34**, **65** and **66** as shown in Fig. 39.

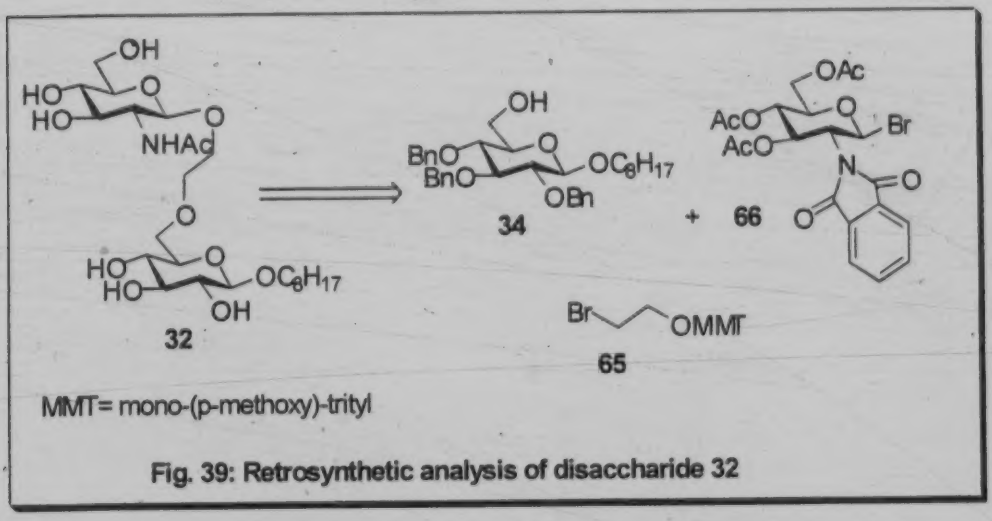


Fig. 39: Retrosynthetic analysis of disaccharide 32

The preparation of **65** required the protection of the alcohol in the commercially available 2-bromoethanol with chloro-p-methoxyphenyldiphenylmethane in pyridine (Fig. 40). Compound **34** was converted into the alkoxide by treatment with NaH, and **65** was slowly added to this mixture. This reaction proved somewhat delicate and required careful monitoring by TLC analysis and addition of **65** until all the alcohol was consumed. Compound **67** was selectively deprotected to liberate the free hydroxyl group, **68**, by stirring in 80% acetic acid.

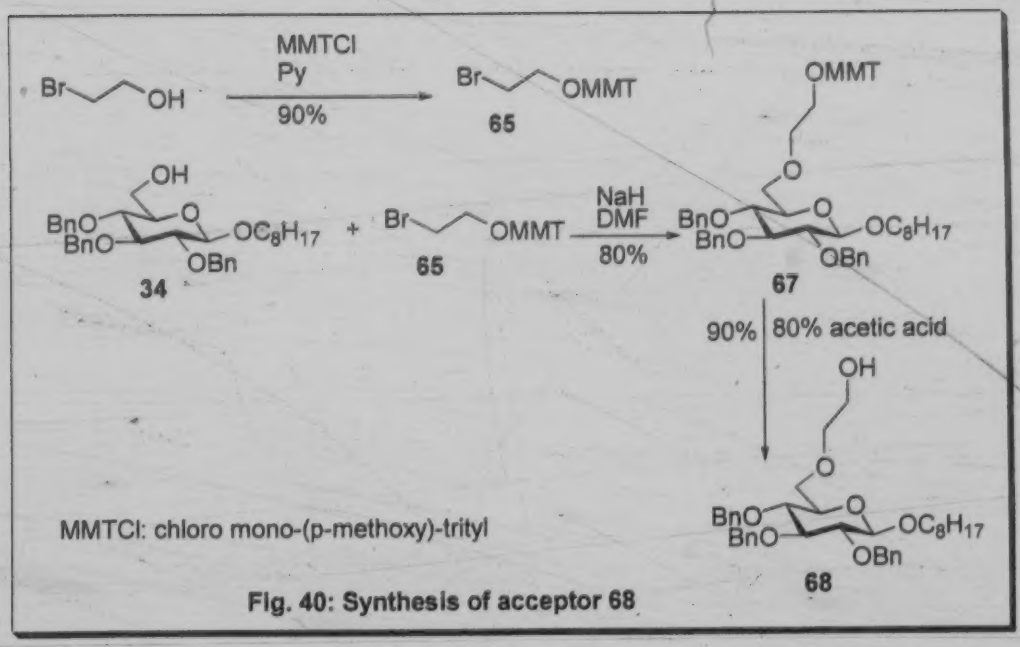


Fig. 40: Synthesis of acceptor 68

The commercially available D-glucosamine hydrochloride was used as a precursor for the synthesis of the donor **66** (Fig. 41)[125]. First of all, the amine group was protected as a phthalimido group in the presence of phthalic anhydride and sodium hydrogen carbonate to give **69**. Acetylation of **69** with acetic anhydride and pyridine furnished the cyclized and acetylated compound **70**. The β bromide **66** was obtained when **70** was treated with 33% hydrogen bromide in acetic acid.

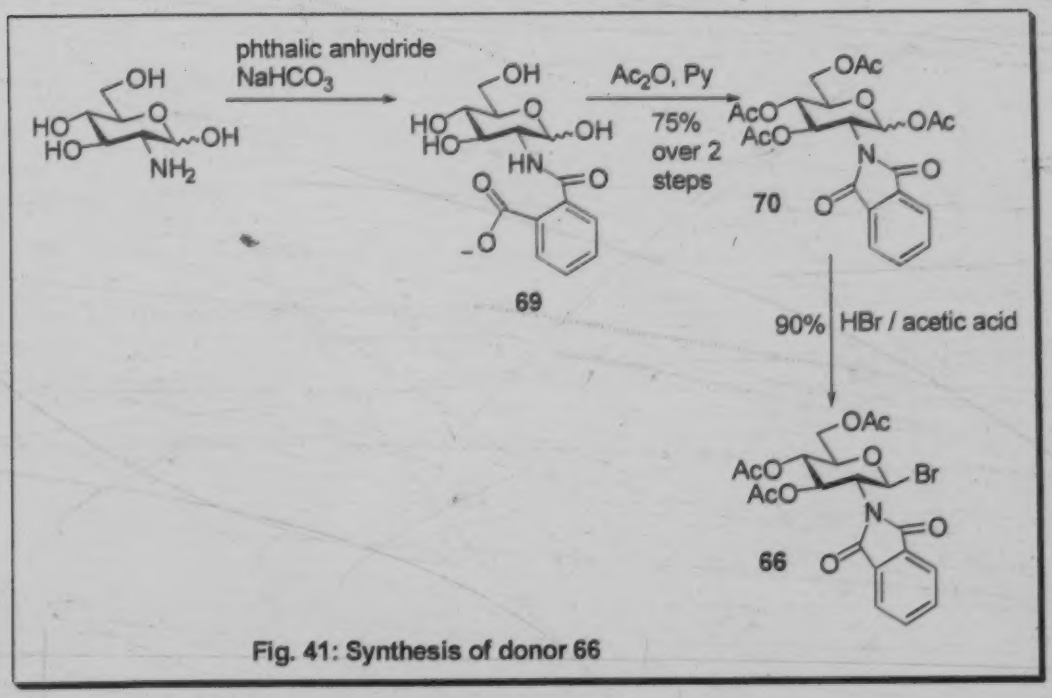


Fig. 41: Synthesis of donor 66

The coupling of **68** with **66** was done in the presence of silver trifluoromethanesulfonate and sym-collidine at -50° C (Fig. 42). The reaction proceeded in very high yield (97%) and the coupling constants of 159.1 and 164.6 Hz between the anomeric carbons and protons revealed a β configuration present at each glycosidic linkage in **71**. Both the phthalimido and acetate groups were removed by refluxing in ethylenediamine and butanol. However this product was not isolated but was directly converted to the tri-O-acetyl-2-N-acetamido sugar **72** so that it could be purified by column chromatography at this stage. Compound **72** was then debenzylated in the presence of Pearlman's catalyst, $Pd(OH)_2/C$, and hydrogen gas to give **73**. Subsequent deacetylation with sodium methoxide afforded the deprotected disaccharide **32**. The anomeric coupling constants of 159.9 and 160.9 Hz are characteristics of the β linkages.

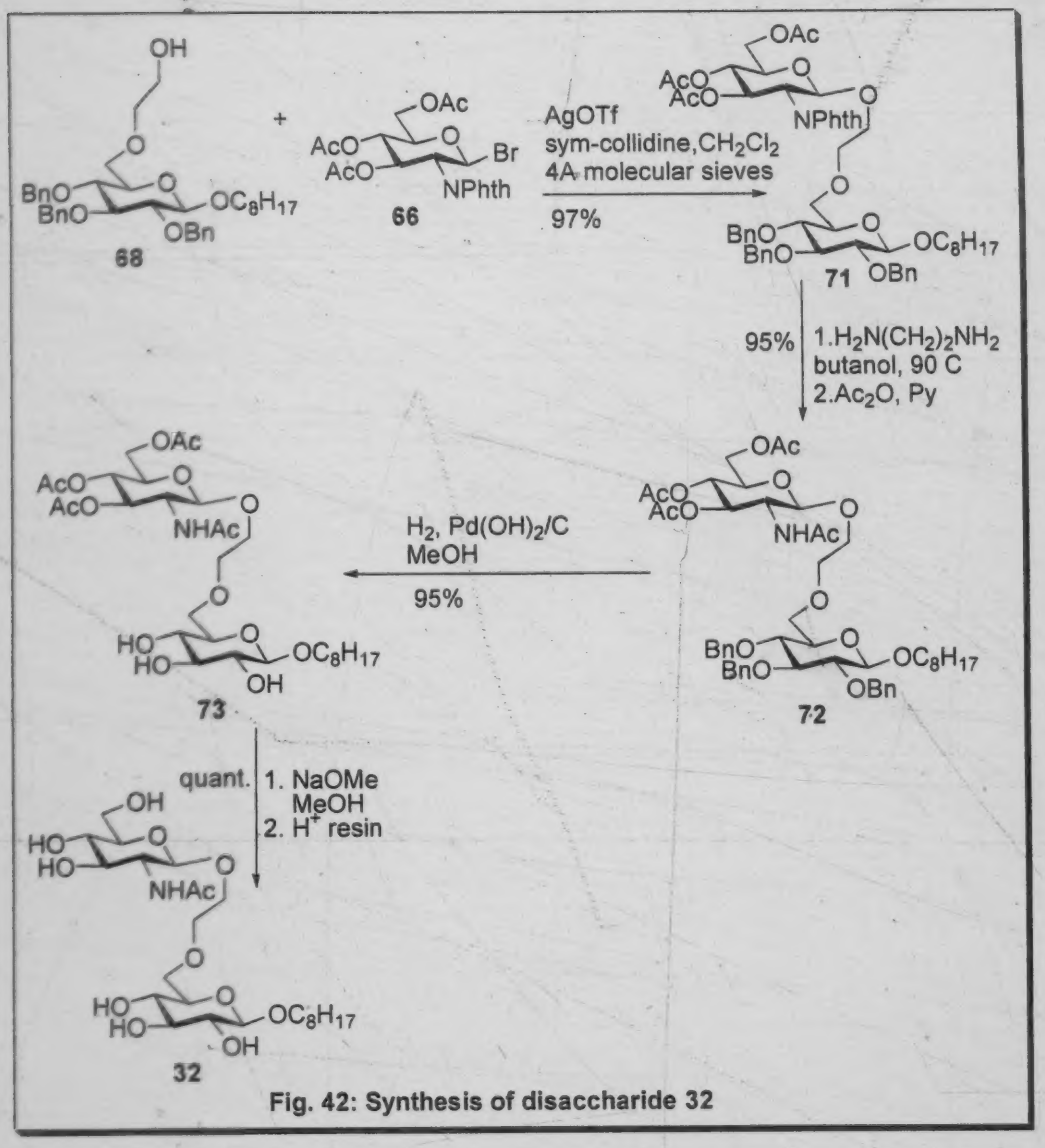


Fig. 42: Synthesis of disaccharide 32

2.3.2 Design and Synthesis of (2-Methoxyethyl) 2-acetamido-2-deoxy-β-D-glucopyranoside (33)

Compound **33** was prepared as a control for **32** (in the event that it will be an inhibitor) showing that the glucose ring is required for activity. The retrosynthetic analysis of compound **33** suggested the use of the two building blocks **66** and 2-methoxy ethanol as shown in Fig. 43.

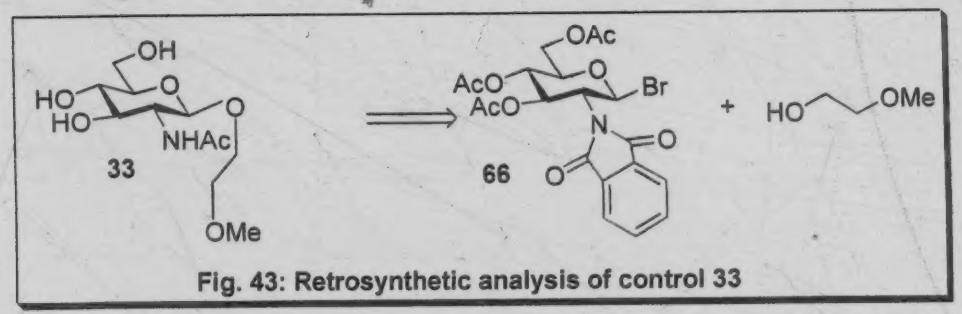


Fig. 43: Retrosynthetic analysis of control 33

The preparation of **33** involved first the coupling of the donor **66** with 2-methoxy ethanol in the presence of tetramethylurea and silver trifluoromethanesulfonate at -30° C to form **74** (Fig. 44). The phthalimido group was then converted to the acetamido functionality by treatment with ethylenediamine in butanol followed by acetylation. Compound **75**, which was formed from the previous reaction, underwent Zemplen deacetylation to remove the acetyl groups resulting in **33**. The anomeric coupling constant of 160.1 Hz showed that indeed a β linkage was formed.

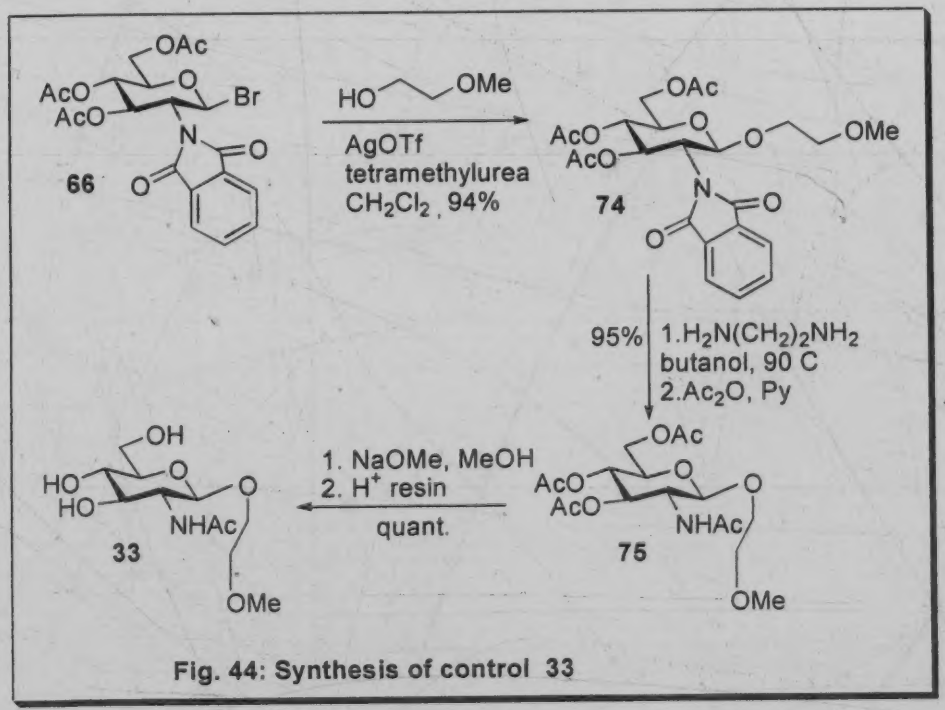


Fig. 44: Synthesis of control 33

Table 1 shows a comparison of the ^{1}H NMR spectra of the final compounds prepared and parent **7**. It can be seen that most of the chemical shifts and coupling constants between the compounds prepared and **7** are nearly identical. However there is a prominent difference at the 2'' position of the terminal residue of about 0.3/0.6 ppm for **31** and **29** respectively. This is due to the presence of oxygen at this position for both compounds compared to nitrogen in parent **7**.

Nucleus	7	29	31	32	33
H-1	4.45(8.0)	4.46(8.0)	4.45(8.0)	4.44(8.0)	
H-2	3.25(9.5)	3.26(7.0)	3.25(8.0)	3.25(9.0)	
H-3	3.5	3.48(2.5)	3.5	3.47(9.0)	
H-4	3.5	3.4	3.5	3.39(9.5)	
H-5	3.6	3.60	3.59	3.5	
H-6a	3.95(11.0,5.0)	3.96(11.5,5.0)	3.93(11.5,5.0)	3.9	
H-6b	3.76	3.81(11.5,2.0)	3.8	3.7	
H-1'	4.89(1.5)	5.03(1.5)	5.01(1.3)		
H-2'	4.11(3.4)	4.18(3.5)	4.14(3.5)		
H-3'	3.83(9.5,3.5)	3.9	3.9		
H-4'	3.52(10.0,9.0)	3.7	3.7		
H-5'	3.6	3.74	3.7		
H-6a'	3.91	3.9	3.9		
H-6b'	3.6	3.7	3.8		
H-1''	4.57(8.5)	4.57(8.0)	4.54(8.0)	4.57(8.5)	4.45(8.5)
H-2''	3.70(10.0,8.5)	3.08(9.0)	3.39(9.0)	3.7	3.62(10.0)
H-3''	3.56(11.0,9.0)	3.5	3.52(9.0)	3.5	3.45(8.5)
H-4''	3.5	3.4	3.57(9.0)	3.5	3.30
H-5''	3.4	3.4	3.9	3.5	3.25(9.5,5.0,2.0)
H-6a''	3.90	3.9		3.9	3.86(12.0,2.0)
H-6b''	3.76	3.7		3.7	3.7
$COCH_3$	2.06			2.05	1.96
CH_2CH_3	0.86(7.0)	0.87(7.0)	0.86(7.0)	0.86(7.0)	

Table 1: Comparison of ^{1}HNMR spectra of final compounds and compound 7
Chemical Shifts (ppm) and Coupling Constants (Hz) are shown. All compounds are in D_2O except compound **33**, which is in CD_3OD. ^{1}HNMR recorded at 500 and 600 MHz.': mannose, '': GlcNAc/Glucuronic residue

Table 2 shows a comparison of the ^{13}C NMR data of the compounds prepared and parent **7**. It can be seen that dramatic differences exist between the chemical shifts at the C-2'' position for the compounds prepared (**29** and **31**) and **7**. This is attributed to the fact that both compounds **29** and **31** have an oxygen atom while compound **7** is attached to a nitrogen atom. Therefore, the C-2'' position of both **29** and **31** is deshielded compared to **7**. There are also small differences between the other positions of the terminal GlcNAc residue. However, these small changes in chemical shifts would not result in different conformations adopted by these compounds. Therefore, it might be anticipated that these compounds have similar conformations to the original acceptor **7** and that this would not affect recognition by the enzyme.

Nucleus	7	29	31	32	33
C-1	103.1(161.1)	103.1(160.8)	103.1(161.2)	103.1(159.9)	
C-2	73.9	74.2	73.9	73.9	
C-3	76.6	76.6	76.8	76.6	
C-4	70.3	70.4	70.4	70.6	
C-5	74.9	74.9	74.8	74.7	
C-6	66.6	66.6	66.7	71.6	
C-1'	97.6(170.1)	98.0(170.9)	98.4(171.5)		
C-2'	77.1	77.6	78.3		
C-3'	70.4	70.4	70.5		
C-4'	68.0	68.0	67.5		
C-5'	73.7	75.8	73.4		
C-6'	62.3	61.8	61.1		
C-1''	100.3(161.7)	101.8(162.1)	102.3(162.2)	102.0(160.9)	102.7(160.1)
C-2''	56.2	83.2	73.2	56.4	57.4
C-3''	74.2	76.9	75.9	75.7	76.2
C-4''	70.7	70.3	72.3	70.7	72.2
C-5''	76.8	73.7	75.9	76.7	78.0
C-6''	61.4	61.5	174.8	61.6	62.8
$COCH_3$	175.6			175.3	173.8
$COCH_3$	23.1			23.1	23.0
CH_2CH_3	14.2	14.3	14.2	14.2	

Table 2: Comparison of ^{13}C NMR spectra of final compounds and compound 7
Chemical Shifts (ppm) and Coupling Constants ($J_{C\text{-}1,H\text{-}1}$) are shown. All compounds are in D_2O except **33**, which is in CD_3OD. Spectra are recorded at 125 MHz. ': mannose, '': GlcNAc/Glucuronic residue

Chapter 3

Enzymatic Assays[1]

3.1 Introduction

This Chapter deals with the enzymatic evaluation of the new active-site probes described in Chapter 2. However, a brief description of the various assays for glycosyltransferases used during the evaluation of new compounds, as acceptors/inhibitors, will be discussed first. Various methods for assaying glycosyltransferase activity are available and including C_{18} Sep-Pak assays[126], HPLC[127], Affinity Chromatography[128], ELISA[129], Particle Exclusion Assay[130] and so on. Usually depending on the characteristics of the acceptors used, some methods are preferred over the others.

3.2 Assays for GlcNAcT-V

During the evaluation of acceptors for N-acetylglucosaminyltransferase V activity, three main assays have been used namely C_{18} Sep-Pak[126], ELISA[129]and Frontal Affinity Chromatography coupled to Mass Spectrometry (FAC/MS)[131]. These three methods of evaluation will be described in the following sections.

3.2.1 C_{18} Sep-Pak Assay

This method for the assay of glycosyltransferase activity was developed by Palcic *et al.* in 1988[126]. The assay depends on the availability of a synthetic glycoside acceptor attached to a hydrophobic aglycone. After the incubation of the enzyme with the synthetic acceptor and the radiolabelled sugar nucleotide, the product formed during the

1: The enzymatic assays were performed by Adam Szpacenko in the laboratory of Professor Monica M. Palcic in the Department of Chemistry, University of Alberta.

reaction can be easily separated from the reaction mixture by adsorption on a C_{18} reverse phase cartridge. The advantage of this assay is that it is very rapid compared to other techniques (which are tedious and time consuming) and no specialized equipment is required. Moreover it has good sensitivity. Fig. 1 shows a schematic representation of the Sep-Pak assay[132].

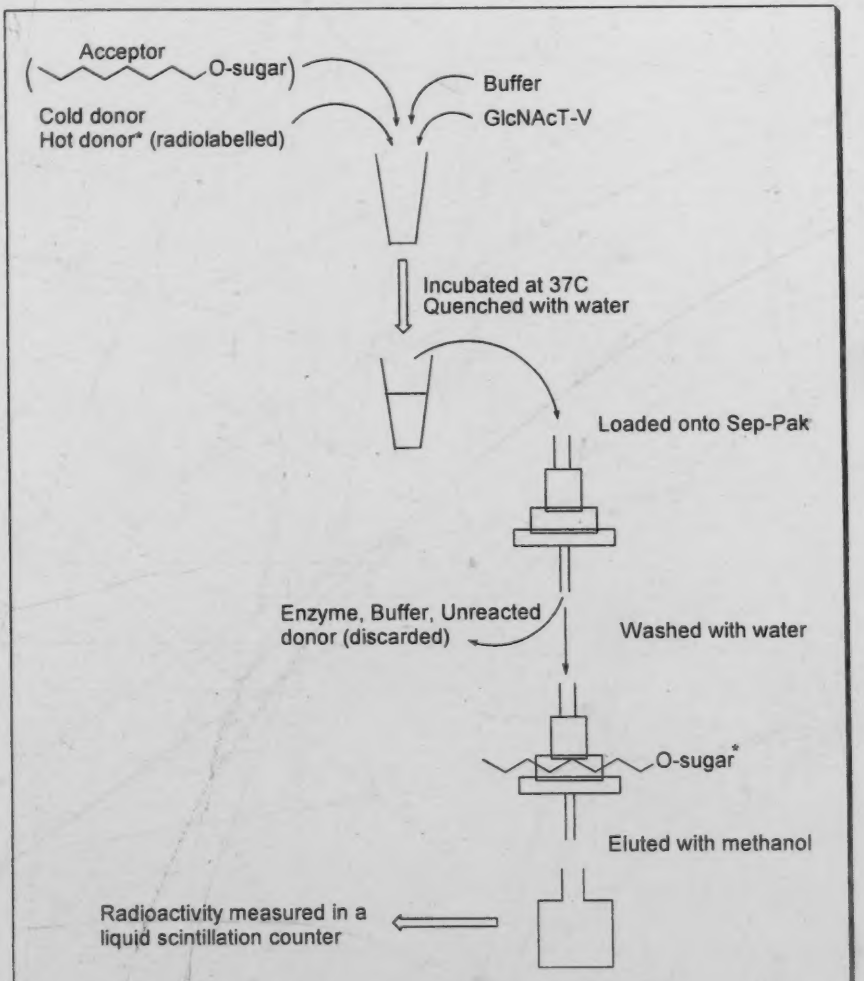


Fig. 45: Schematic representation of a Sep-Pak assay

The general procedure employed during this assay involves the following steps[33,126,133]. Incubations are usually carried out in small plastic scintillation vials since

the enzymes are inactivated on glass surfaces. The synthetic acceptor, the sugar nucleotide, a source of the enzyme and the sugar nucleotide (radiolabelled in the sugar moiety) are added to the reaction vial. The sugar nucleotide is added in excess to make sure that the reaction goes towards completion as it can be degraded by metal cations present in the reaction mixture. An assay buffer containing sodium cacodylate, EDTA, glycerol and bovine serum albumin (BSA) are also added. The EDTA complexes with any metal ion present in the reaction so that there is no interference from any other glycosyltransferases that usually require a divalent metal cation for reaction. The BSA and glycerol are added to the glycosyltransferase reactions to stabilize the enzyme during long reactions. It should be noted that during these reactions both the substrate and products could inhibit the rate of the reaction. For example, nucleotides, which are formed as side products during the reaction, can be potent inhibitors of the enzyme. Therefore alkaline phosphatase, which hydrolyses the nucleotides to the corresponding nucleosides, is added to the reaction mixture.

Incubations are generally carried out at 37° C and it is advisable to carry out a full kinetic analysis by varying substrates concentrations to determine V_{max} (maximal rate of reaction at saturating substrate) and K_m. Consequently, the amount of enzyme required for completion of the reaction can be determined. Enzyme activity is defined in Units (U). One Unit is the amount of enzyme producing 1μmol of product per minute at 37° C. Therefore, the rate of the reaction can also be estimated from such analysis. In preparative reactions, the progress of the reaction is monitored by thin layer chromatography. Small aliquots can also be removed and analysed after some interval of time throughout the reaction. The pH of the solution should be verified during the reaction, as it tends to drop due to the production of phosphoric acid from the action of the alkaline phosphatase.

Once the reaction is complete, the reaction mixture is quenched by dilution with water and loaded onto a C_{18} Sep-Pak cartridge. The Sep-Pak cartridge is previously

conditioned by washing with methanol followed by water. Due to the presence of the hydrophobic aglycone, the radiolabelled product (along with non-labelled unreacted acceptor) is adsorbed onto the C_{18} cartridge while the unreacted sugar nucleotide and by-products are easily removed by washing with water. The water solutions are examined until constant background counts (disintegration per minute, DPM) are obtained to ensure that all unreacted sugar nucleotide has been removed. The radiolabelled reaction product is then eluted with methanol, concentrated and dissolved in water. An ACS liquid scintillation cocktail is added and the solution is quantitated by liquid scintillation counting using a Beckman LS 1801 scintillation counter.

This assay technique has been widely used for the evaluation of inhibitors for GlcNAcT-V. Previous work on this assay showed that eight carbons in the aglycone is sufficient to allow a quantitative enzyme assay and henceforth, researchers prefer to prepare inhibitors as their octyl glycosides.

3.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

In 1990, an enzyme-linked immunosorbent assay for N-acetylglucosaminyltransferase V was developed by Crawley *et al.*[129]. To set up the assay, the trisaccharide acceptor is first covalently attached to bovine serum albumin to form conjugates. Next, the BSA-conjugate of the product that is formed by the addition of GlcNAc to the acceptor is prepared. Rabbits are then immunized with the BSA-conjugate of the tetrasaccharide product. The antiserum raised against the product is then depleted of antibodies that cross-react with the acceptor-BSA conjugate by passage through an affinity column of immobilized acceptor. This leaves an antiserum containing antibodies that bind to the product, but not to the acceptor.

For the assay, microtitre plates are coated with the BSA-conjugate of the acceptor, and then incubated with a source of the enzyme, GlcNAcT-V, and

UDP-GlcNAc. The product that is formed is then detected and quantified using the refined rabbit antiserum. Antibodies bound to the product on the plate are quantitated using anti-antibodies to which alkaline phosphatase are covalently attached. Addition of p-nitrophenyl phosphate to the wells then results in hydrolysis of the phosphate ester, and the resulting yellow colour is proportional to the amount of product that was formed.

The benefits of this assay technique include the simultaneous identification of the product and its superior sensitivity over radiochemical assay. However, a major drawback of this assay is the requirement of both the acceptor and product, which are obtained by tedious multistep chemical synthesis.

3.2.3 Frontal Affinity Chromatography Coupled to Mass Spectrometry (FAC/MS)

Finally, frontal affinity chromatography coupled to mass spectrometry has been used to screen mixtures of inhibitors[131]. In this technique, streptavidin is immobilized onto a column. Biotinylated GlcNAcT-V is then adsorbed onto the streptavidin and a sample containing the potential inhibitors (or acceptors) is continuously injected into the column. The order of elution relates the binding affinity of the inhibitor to the enzyme with the tightest inhibitor eluting last. This technique has the advantage of rapidly screening a library of compounds ranking them according to their affinities. The binding parameters are then calculated. The only disadvantage of this technique is that inhibitors must have different molecular masses to differentiate between them since mass spectrometry is used as the method of detection.

3.3 Enzymatic Evaluation of the New Inhibitors

The activity of the synthetic compounds described in Chapter 2 were determined using the conventional Sep-Pak radioactive assay technique[126] whereby the rate of transfer of ^{3}H-labelled UDP-GlcNAc from the commercially available sugar nucleotide to

the substrate was quantitated. The same procedure as the one described in section 3.2.1 was used. The concentration of the acceptor was 0.60 mM while the UDP-GlcNAc was 0.25 mM. The assay buffer consisted of 25 mM sodium cacodylate, 5 mM EDTA, 10% (v/v) glycerol and 0.5 mg/ml BSA and had a pH of 6.55. Hot radioactive ^{3}H-UDP-GlcNAc was added and the enzyme was obtained from human serum whereby blood was allowed to clot at room temperature, refrigerated overnight at 4° C and centrifuged to remove red blood cells[129]. The total volume of the assay mixture was 20 μL and incubation time was 31 minutes. The activity of the compounds were compared to the control acceptor, octyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside **7**. The results from the evaluation of **29**, **31**, **32** and **33** as substrates are shown in Table 3. The structures of the compounds used are shown in Fig. 24.

Compound	% Activity compared to control
29 (OMe)	1.79
31 (COO^-)	1.66
32 (disac)	1.14
33 (monosac)	1.32

Table 3: Evaluation of compounds as substrates for GlcNAcT-V
Concentration = 600 mM, UDP-GlcNAc = 0.25 mM, reaction time =31 min

All of the analogs showed barely detectable acceptor activities, exhibiting only the background counts in the experiment. In order to verify if the compounds were perhaps very weak acceptors, the assay was repeated with the two compounds, **29** and **31**, which had the highest probability of acting as acceptors since these contained the reactive mannose residue. The concentration of the enzyme was increased (5×) and the reaction time was extended to 2 hours. However once again, very low activity was observed

confirming that the compounds were effectively not acting as substrates.

No activity was expected for **32** and **33** since the reactive 6'-hydroxy group is absent in both compounds so that they cannot react with the enzyme. However, the other two compounds **29** and **31** were both inactive even though they could potentially react with the UDP-GlcNAc. Despite the fact that the compounds failed to act as substrates, important recognition information could be obtained from this study.

Analogs of **7**, whereby NH_2 and OH replaced the 2''-acetamido group respectively, were both found to be good substrates for the enzyme[107,108]. However **29**, which has a methoxy group, turned out to have no activity towards the enzyme. Various explanations could account for this lack of activity of **29**. Firstly, the methoxy substituent, being a bulkier group compared to the hydroxy and amino groups, could suffer from steric hindrance. However, the acetamido group is even bigger than the methoxy group so that the most plausible explanation for this outcome would be because of hydrogen bonding. All the groups found at the 2'' position previously (NHAc, NH_2, OH) are all capable of hydrogen bonding, while hydrogen bonding would be impaired in **29**. Therefore the lack of activity of **29** might be ascribed to loss of hydrogen bonding. Thus, hydrogen bonding at the 2'' position of the GlcNAc ring may be a critical recognition element for the enzyme active site. It can be concluded that the GlcNAc ring tolerates changes at the 2'' position to other substituents as long as there is the potential for hydrogen bonding. Finally another difference between **29** and the original acceptor **7**, is that the methoxy group can spin around the C-O bond while rotation cannot occur in the acetamido group. Therefore, this bulky spinning group could result in a different relative orientation of the mannose unit containing the reactive 6'-OH group so that it is no longer recognized by the enzyme. However analysis of the ^{1}H NMR and ^{13}C NMR spectra showed no great differences in chemical shifts and coupling constants which would be a result of different conformations.

The lack of activity of **31** can be easily ascribed to two factors: unfavourable

repulsion and impaired hydrogen bonding. As discussed earlier, **31** being negatively charged will suffer from unfavourable repulsion by negatively charged amino acids (Asp or Glu) present in the active site. Therefore an attractive hypothesis would be that there is, in the vicinity of the 6''-OH group, a negatively charged amino acid residue that prohibits recognition of negatively charged substrates due to strong electrostatic repulsion of two negatively charged groups in that area. Furthermore, it could be postulated that the trisaccharide acts as a hydrogen donor during hydrogen bonding to the enzyme. Therefore, **31**, which does not possess an electropositive hydrogen, cannot act as a hydrogen donor anymore. Recognition by the enzyme is therefore lost.

All four compounds were next evaluated as inhibitors for GlcNAcT-V. The assay conditions for the reaction were acceptor **7** (0.0375 mM), UDP-GlcNAc (0.25mM), inhibitor (0.6 mM) and the assay buffer consisted of 25 mM sodium cacodylate, 5 mM EDTA, 10% (v/v) glycerol and 0.5 mg/ml BSA. The total volume of the assay mixture was 20 μL at a pH of 6.55 and incubation time was 21 minutes. The result from the evaluation of **29**, **31**, **32** and **33** as inhibitors is shown in Table 4.

Compound	Activity (dpm)	% Inhibition	Type of inhibition
7 (acceptor)	2950	-	-
29 (OMe)	2089	29.2	competitive
31 (COO^-)	3009	~ 0	
32 (disac)	3009	~ 0	-
33 (monosac)	2482	15.8	competitive

Table 4: Evaluation of compounds as inhibitors for GlcNAcT-V

Concentration of acceptor (7) = 0.0375mM, inhibitor = 0.6 mM, UDP-GlcNAc= 0.25 mM, reaction time = 21 min

From the result it can be seen that in the absence of any inhibitors, the activity observed with only acceptor **7** is 2950 dpm. Therefore, since the addition of **29** results in a lower activity, it behaves as a competitive inhibitor with an inhibition of 29.2 %. The kinetic analysis of this compound shows that apparent K_m (K_{mapp}) increases for acceptor **7**. A hyperbolic curve was obtained when K_{mapp} was plotted against different concentrations of inhibitor. However, a surprising and unusual result was that it also acts as an activator since V_{max} increases. This shows that the compound can at least bind to the enzyme however the transfer is not possible because of the absence of some important recognition factor during the E-S complex.

Compound **31** was not an inhibitor since nearly similar activity was observed compared to the control. This shows that unfavorable repulsion between similarly charged groups precludes binding to the enzyme.

It was rather disappointing that **32** did not act as an inhibitor. Both the three hydroxyl groups and the ring oxygen of the mannose ring have been shown not to be important for recognition by the enzyme[90,97,100,101]. However this study clearly demonstrates that some hydrophobic interaction from the mannose ring is required during binding to the enzyme.

Compound **33** exhibited a rather strange behaviour as it had an inhibition of 15.8 %. Since the key polar groups are absent in **33**, it was expected to have no effect. It is rather difficult to explain this result because there is no possibility that it might be contaminated by any trisaccharide during the preparation of **33**.

Chapter 4

Conclusion

4.1 Summary

N-acetylglucosaminyltransferase V is a key enzyme in the biosynthesis of highly branched asparagine-linked oligosaccharides. Recent years have seen an increased interest in this enzyme as a strong correlation between the activity of the enzyme and its involvement in cancerous tissues was observed. An increased activity of this enzyme was found in transformed cells and specific increase in its activity has been correlated with the metastatic potential of both rodent and human tumor cells[13-18]. Thus, this enzyme has become a target for the development of glycosyltransferase inhibitors, which could have the potential for reversing the metastatic behaviour of tumor cells. Work from this laboratory has shown that the trisaccharide octyl 2-O-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside **7**, is an excellent acceptor for the enzyme[89].

Analogs of this trisaccharide were designed to probe the active site of the enzyme. Compounds **29**, **31**, **32** and **33** were prepared by chemical synthesis while the attempted synthesis of octyl 2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside **30** failed. The structures of the compounds are shown in Fig. 46. The synthesis of the compounds involved conventional coupling reactions in the presence of silver trifluoromethanesulfonate for the coupling reactions involving the bromides and trimethylsilyltriflate for the coupling of an imidate. All the reactions were high yielding. However, the crucial deoxygenation reaction failed during the attempted synthesis of **30**, so it could not be prepared.

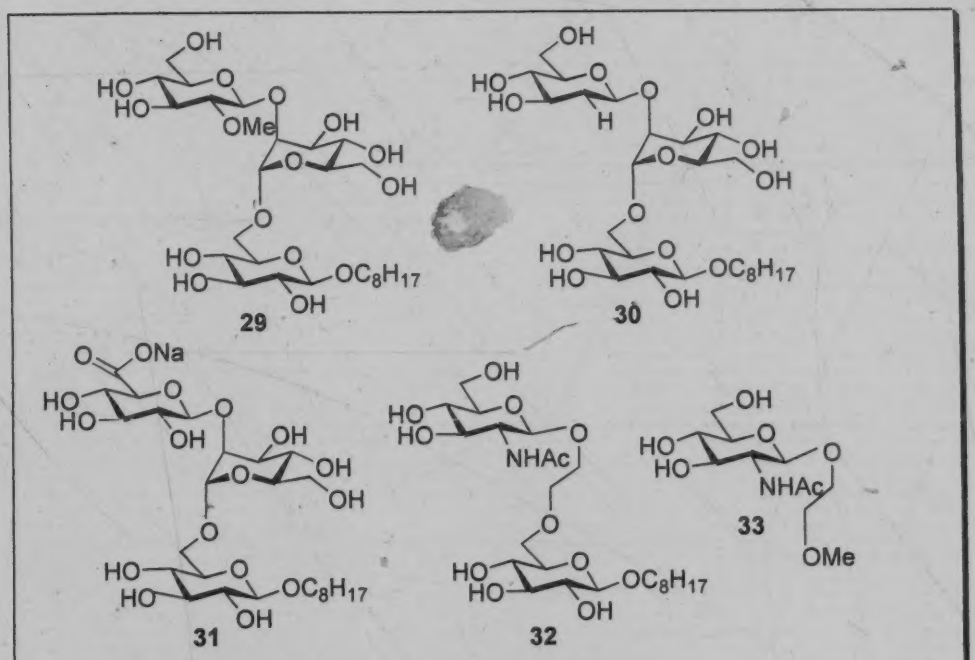


Fig. 46: Summary of compounds designed

All the compounds were then evaluated as both substrates and inhibitors for the enzyme GlcNAcT-V. However, none of them were substrates at a concentration of 0.6 mM. Only compound **29** showed moderate inhibition (29.2% of control). However, an interesting observation made during the kinetic analysis of compound **29** was that V_{max} increases showing that it acts as an activator. This rather unusual behaviour is difficult to explain and the effect of allostery might be a possible explanation.

Even though none of the compounds were good substrates/inhibitors, useful information about the enzyme active site could be obtained. Important hydrogen bonding may be required for recognition at the 2'' position of the N-acetylglucosamine ring. The enzyme tolerates substitution at that position as long as there is the possibility for hydrogen bonding. Furthermore, the amino acids around the 6'' position on the N-acetylglucosamine ring are either negatively charged or they act as hydrogen-bond

acceptors. This analysis was made after observing that compound **31** had no activity towards the enzyme. Thus, the activity of compound **31** was impaired as it is negatively charged and lacks an electropositive hydrogen atom.

Another conclusion made after analysing that compound **32** had no activity towards the enzyme is that a hydrophobic interaction from the mannose ring is required for recognition by the enzyme. Fig. 2 shows a schematic representation of the groups required for recognition by the enzyme.

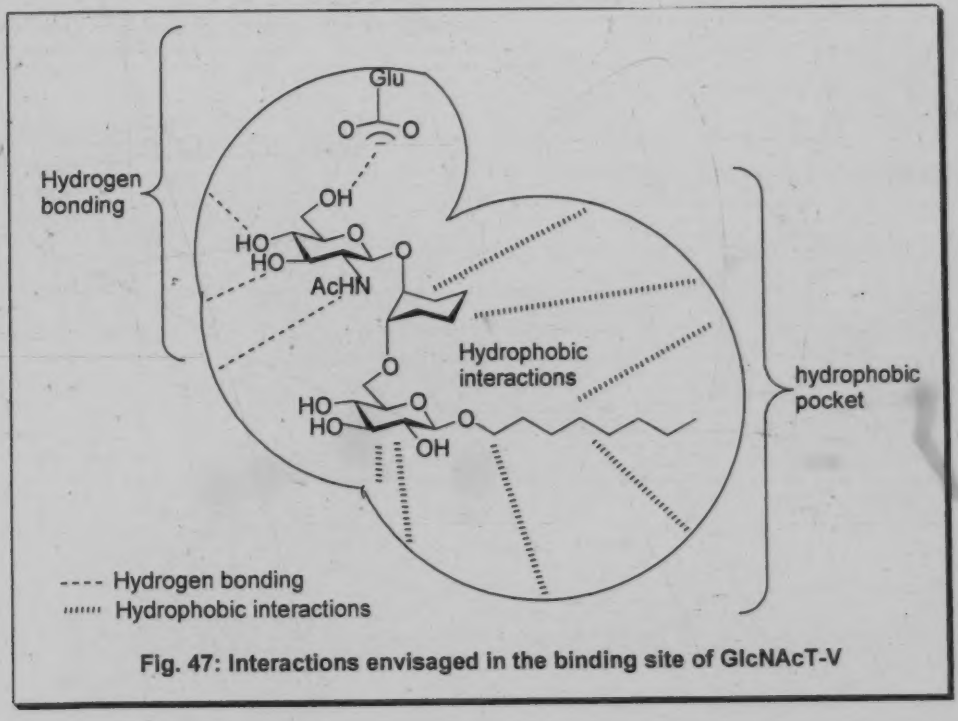


Fig. 47: Interactions envisaged in the binding site of GlcNAcT-V

Usually the only disadvantage of using carbohydrate compounds as inhibitors is their delivery inside cells. Being polar in nature, they cannot penetrate the lipid bilayer resulting in no effect. Therefore, researchers aim towards the preparation of hydrophobic analogs. However carbohydrate inhibitors can still act on secreted GlcNAcT-V preventing metastasis.

4.2 Future work

Based on this study, an attractive analog of the trisaccharide 7 would involve replacement of the mannose ring by a cyclohexyl ring and this would address the question about whether this moiety is enough for the required hydrophobic interaction. However the synthesis of this analog would be very challenging, as it requires the cyclohexyl ring to have the two sugar moieties in the diaxial position. Most probably a tertiary butyl group should be incorporated in this cyclohexyl ring so as to avoid flipping to the more stable diequatorial position.

Since chemical synthesis of trisaccharide analogs is very tedious and time consuming, Combinatorial Chemistry seems an attractive alternative for the preparation of glycosyltransferase inhibitors. Indeed, work is currently in progress in this laboratory targeting the synthesis of large libraries of inhibitors for GlcNAcT-V. This mixture of compounds can be easily assayed using the FAC/MS assay technique whereby two aims will be accomplished at the same time. Firstly, an excellent inhibitor could be detected from this large library of compounds and secondly the sensitivity of the FAC/MS assay method could be demonstrated. Therefore, future work on the synthesis of inhibitors for GlcNAcT-V should aim at producing libraries of compounds instead of individual compounds.

Efforts should be directed at producing a large amount of the enzyme with higher purity in the hope of having a crystal structure, which would shed light on the structure of the active-site of this important enzyme.

Chapter 5

Experimental

General methods:

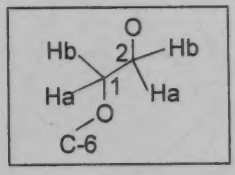
Unless otherwise specified, all commercial reagents were used as supplied. Solvents that are described as "dry" were distilled from still pots containing appropriate drying agents. Aqueous solutions for work-up procedures were made with deionized water; aqueous solutions for reactions or chromatography were made with MilliQ water. Crushed molecular sieves (BDH Inc.) were activated for use by flaming in a round bottom flask under vacuum. Amberlite® IR 120 and Amberlite® IRC 50 exchange resin (H^+) were from Caledon Laboratories Ltd. and Aldrich Chemical Company Inc. respectively. Both resins were washed with methanol prior to use.

Analytical thin layer chromatography (TLC) was performed on silica gel 60-F_{254} from EM Science/Merck. TLC detection was achieved by fluorescence quenching under UV light, staining with ninhydrin, charring with 5% sulphuric acid in ethanol, or staining with cerium molybdate. Column chromatography was performed on silica gel 60 (230-400 mesh) from SiliCycle or Rose Scientific Ltd. Reverse-phase Sep-Pak (C-18) cartridges were from Waters Associates. Millex-GV (0.22 μM) filter units were from Millipore.

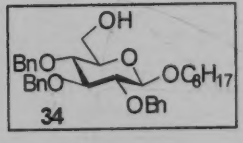
Mass spectra were obtained from the Departmental Mass Spectrometry Laboratory. ^1H NMR spectra were acquired on 300, 400, 500, or 600 MHz Varian spectrometers. ^{13}C NMR spectra were acquired on 400 or 500 MHz Varian spectrometers. All experiments were performed at 27 °C. Proton chemical shifts are referenced to an internal standard at δ 7.24 ppm for solutions in $CDCl_3$, δ 3.30 ppm for solutions in CD_3OD, or to 0.1% external acetone at δ 2.23 ppm for solutions in D_2O.

Carbon-13 chemical shifts are referenced to an internal standard at 77.0 ppm for solutions in $CDCl_3$, 49.0 ppm for solutions in CD_3OD, or to 1% external acetone at 31.1 ppm for solutions in D_2O. In most cases, carbon-13 NMR data were obtained from APT experiments performed on a 500 MHz spectrometer. Proton and carbon assignments were made after analysing the GCOSY, GTOCSY and HMQC spectra.

Protons and carbons of the ethyl group at the 6-position of the glucose ring present in compounds **67**, **68**, **71**, **72**, **73**, **32** are designated as shown below, and were not specifically assigned.



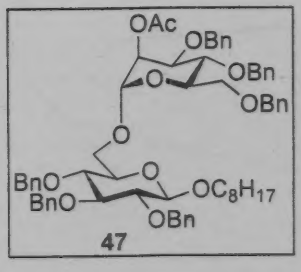
Octyl 2,3,4-tri-O-benzyl-β-D-glucopyranoside (**34**)



Octyl β-D-glucopyranoside (1.43 g, 4.88 mmol) was dissolved in anhydrous pyridine (15 ml) and added to chloro-4-methoxy phenyldiphenylmethane (MMT-Cl, 2.26 g, 7.32 mmol). The resulting yellow solution was stirred for 24 h. The mixture was then concentrated and it was subjected to column chromatography using CH_2Cl_2-MeOH (9:1). The eluate containing the product (R_f 0.44 in 9:1 CH_2Cl_2: MeOH) was concentrated and the residue was benzylated with NaH (742 mg, 31 mmol) and benzyl bromide (2.2 ml, 18 mmol) in DMF (50 ml) for 16 h. The solution was then quenched with methanol and the pH was checked to make sure it was basic. After evaporation of methanol, ethyl acetate was added to the solution and it was sequentially washed with water and brine and dried over sodium sulfate. The solution was filtered to remove the drying agent. Evaporation of the solvent gave a residue which was purified by column chromatography using hexane-

EtOAc (8:1). The fractions containing the product were concentrated. The residue was then dissolved in 80% aq. acetic acid (100 ml) and it was stirred for 20 h. After evaporation of the solvent, the residue was purified by column chromatography (hexane-EtOAc 4:1) to give **34** (R_f 0.55 in hexane-EtOAc 3:1, 1.37 g, 50%) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.34-7.22 (m, 15 H, Ar-H), 4.95-4.60 (6×ABq, 6 H, J_{gem} 11 Hz, PhC$\underline{H_2}$O), 4.42 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 3.90 (dt, 1 H, J_{gem} 9.5 Hz, J_{vic} 6.5 Hz, octyl C1-Ha), 3.85 (dd, 1 H, J_{gem} 12 Hz, J_{vic} 2.5 Hz, H-6a), 3.69 (dd, 1 H, J_{gem} 12 Hz, J_{vic} 5 Hz, H-6b), 3.65 (t, 1 H, $J_{3,2}$ 9.0 Hz, H-3), 3.57-3.50 (m, 2 H, H-4, octyl C1-Hb), 3.40 (dd, 1 H, $J_{2,3}$ 9.0 Hz, $J_{1,2}$ 7.5 Hz, H-2), 3.35 (dd, 1 H, $J_{5,4}$ 10.0 Hz, $J_{5,6b}$ 5.0 Hz, $J_{5,6a}$ 2.5 Hz, H-5), 1.90-1.83 (bs, 1 H, O-H), 1.67-1.59 (m, 2 H, octyl C2-H), 1.42-1.19 (m, 10 H, 5 octyl CH_2), 0.86 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 138.8, 138.7, 138.3 (quaternary Ph), 128.8, 128.7, 128.6, 128.3, 128.2, 128.1, 127.9 (Ar-H), 104.0 (C-1, $J_{C\text{-}1,H\text{-}1}$ 157.9 Hz), 84.8 (C-3), 82.6 (C-2), 77.9 (C-4), 75.9 (C-5), 75.3, 75.1 ($PhCH_2$), 70.7 (octyl C1), 62.4 (C-6), 32.1, 30.1, 29.7, 29.5, 26.4, 22.9 (octyl C2-C7), 14.3 (octyl CH_3); HR MS (ES) calcd. for $C_{35}H_{46}O_6Na$ (M + Na) 585.3187, found *m/z* 585.3184.

Octyl 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**47**)

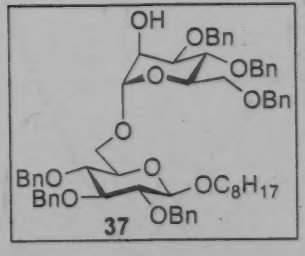


47

A solution of 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannopyranosyl chloride **35** (186 mg, 0.36 mmol) in CH_2Cl_2 (1 ml) was added dropwise to a stirred mixture of **34** (136 mg, 0.24 mmol), silver trifluoromethanesulfonate (86 mg, 0.34 mmol), and 1,1,3,3-tetramethyurea (53 μL, 0.44 mmol) in CH_2Cl_2 (1 ml). After 2 h, the mixture was diluted with CH_2Cl_2 (18 ml), and sym-collidine (44 μL, 0.33 mmol) followed by silver trifluoromethanesulfonate (85 mg, 0.33 mmol) were added to destroy any excess of

glycosyl chloride. After 0.5 h, Et_4NBr (70 mg, 0.33 mmol) was added to precipitate any excess silver, and the solution was then filtered to remove solids. The filtrate was washed twice with saturated aqueous sodium hydrogen carbonate (36 ml) followed by water (36 ml). Then the solvent was evaporated and the residue was purified by column chromatography in hexane-EtOAc 7:2 to give **47** (R_f 0.52 in hexane-EtOAc 2:1, 211 mg, 85 %) as an oil. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.00 (m, 30 H, Ar-H), 5.42 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 1.5 Hz, H-2'), 4.97-4.81 (4×ABq, 4 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.90 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.78-4.62 (4×ABq, 4 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.54-4.40 (2×ABq, 4 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.35 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.93 (ddd, 1 H, J 9.0 Hz, $J_{3',2'}$ 3.5 Hz, J 1.5 Hz, H-3'), 3.91-3.84 (m, 2 H, octyl C1-Ha, H-4'), 3.81-3.76 (m, 2 H, H-6a, H-5'), 3.72-3.57 (m, 4 H, H-6a', H-6b, H-3, H-6b'), 3.48-3.38 (m, 4 H, H-2, octyl C1-Hb, H-4, H-5), 2.14 (s, 3 H, OAc), 1.62-1.48 (m, 2 H, octyl C2-H), 1.39-1.18 (m, 10 H, 5 octyl CH_2), 0.86 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.2 (C=0), 138.6, 138.5, 138.2, 138.0, 137.9 (quaternary Ph), 128.4, 128.3, 128.2, 128.1, 127.9, 127.7, 127.6, 127.5, 127.4 (Ar-H), 103.6 (C-1, $J_{C-1,H-1}$ 158.1 Hz), 97.9 (C-1', $J_{C-1',H-1'}$ 172.9 Hz), 84.9 (C-3), 82.4 (C-2), 77.9 (C-4, C-3'), 75.7, 75.1, 75.0, 74.8, 73.4, 71.7 (6×$PhCH_2$), 74.3 (C-4'), 74.1 (C-5'), 70.1 (octyl C1), 68.8 (C-6'), 68.5 (C-2'), 66.2 (C-6), 32.0, 29.9, 29.6, 29.4, 26.4, 22.8 (octyl C2-C7), 14.3 (octyl CH_3); HR MS (ES) calcd. for $C_{64}H_{76}O_{12}Na$ (M + Na) 1059.5234, found *m/z* 1059.5237.

Octyl 3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**37**)

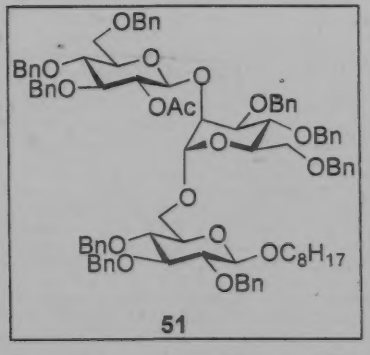


37

Compound **47** (1.40 g, 1.37 mmol) was dissolved in methanol (10 ml) and a catalytic amount of sodium methoxide was added. The solution was stirred for 5 h when TLC showed complete conversion of the acetate group. It was then acidified

with Amberlite IRC 50 (H^+) resin and stirred for 10 min. After filtration, the solvent was evaporated and the residue was subjected to column chromatography in toluene-EtOAc 10:1 to give **37** (R_f 0.47 in hexane-EtOAc 2:1, 1.36 g, quant.) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.20-7.05 (m, 30 H, Ar-H), 4.96 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.95-4.42 (12×ABq, 12 H, J_{gem} 12.0 Hz, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.33 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.06 (bs, 1 H, H-2'), 3.90-3.79 (m, 4 H, octyl C1-Ha, H-3', H-4', H-6a), 3.75 (bm, 1 H, H-5'), 3.71 (dd, 1 H, J 11.5 Hz , J 1.8 Hz, H-6b), 3.65 (dd, 1 H, J_{gem} 10.5 Hz , $J_{6a',5'}$ 4.5 Hz, H-6a') 3.62 (t, 1 H, J 9.0 Hz, H-3), 3.58 (dd, 1 H, J_{gem} 10.5 Hz , $J_{6b',5'}$ 1.8 Hz, H-6b'), 3.48- 3.41 (m, 2 H, H-4, octyl C1-Hb), 3.40-3.35 (m, 2 H, H-2, H-5), 2.34 (bs, 1 H, O-H), 1.61-1.53 (m, 2 H, octyl C2-H), 1.37-1.19 (m, 10 H, 5 octyl CH_2), 0.85 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 138.6, 138.5, 138.3, 138.1, 137.8 (quaternary Ph), 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5 (Ar-H), 103.5 (C-1), 99.5 (C-1'), 84.7 (C-3), 82.3 (C-2), 79.8 (C-3'), 77.9 (C-4), 75.7, 74.9, 74.7, 73.3, 71.8 (6×$PhCH_2$), 74.2 (C-4', C-5), 71.0 (C-5'), 70.1 (octyl C1), 68.8 (C-6'), 68.1 (C-2'), 65.9 (C-6), 31.8, 29.7, 29.4, 29.2, 26.2, 22.6 (octyl C2-C7), 14.1 (octyl CH_3); HR MS (ES) calcd. for $C_{62}H_{74}O_{11}Na$ (M + Na) 1017.5123, found *m/z* 1017.5122

Octyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**51**)

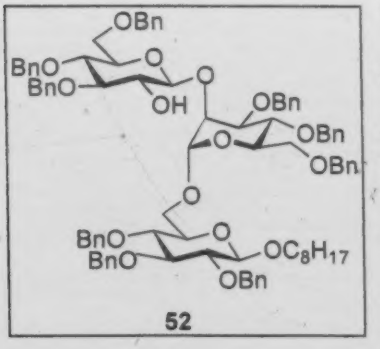


51

Compound **37** (438 mg, 0.44 mmol) was dissolved in dry dichloromethane (5 ml) and silver trifluoromethanesulfonate (283 mg, 1.10 mmol), molecular sieves 4 Å (1 g) and collidine (46 μL, 0.35 mmol) were added to the solution. It was then cooled to -78° C and a solution of 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranosyl bromide **36** (520 mg, 0.935 mmol) in CH_2Cl_2 (0.8 ml) was added

dropwise under argon. The mixture was allowed to warm up to r.t within an h. Excess tetraethylammonium chloride (156 mg, 0.941 mmol) was added and the mixture was stirred for an additional 30 min. The mixture was then diluted with CH_2Cl_2 and filtered through Celite. The filtrate was washed sequentially with 0.5 % HCl, saturated sodium bicarbonate and water. It was dried over sodium sulfate and the resulting solution was filtered and concentrated. Column chromatography of the residue was performed using hexane-EtOAc 7:1 to provide **51** (R_f 0.34 in hexane-EtOAc 3:1, 368 mg, 57%) as a syrup. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.04 (m, 45 H, Ar-H), 5.14 (dd, 1 H, $J_{2'',1''}$ 8.0 Hz, $J_{2'',3''}$ 8.5 Hz, H-2''), 4.86 (3×ABq, 3 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.85 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.84-4.45 (12×ABq, 12 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.44 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.36 (3×ABq, 3 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.34 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.17 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 1.5 Hz, H-2'), 3.93-3.84 (m, 2 H, octyl C1-Ha, H-6a), 3.81 (dd, 1 H, $J_{3',4'}$ 9.0 Hz, $J_{3',2'}$ 3.5 Hz, H-3'), 3.77-3.57 (m, 11 H, H-6a', H-6b, H-6a'', H-6b'', H-5'', H-4'', H-4, H-3'', H-4', H-6b', H-3), 3.55-3.42 (m, 2 H, H-5', octyl C1-Hb,), 3.42-3.34 (m, 2 H, H-5, H-2,), 1.93 (s, 3 H, OAc), 1.64-1.51 (m, 2 H, octyl C2-H), 1.39-1.16 (m, 10 H, 5 octyl CH_2), 0.85 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 169.4 (C=O), 138.8, 138.6, 138.5, 138.4, 138.3, 138.2, 138.1, 138.0, 137.9 (quaternary Ph), 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 127.2 (Ar-H), 103.7 (C-1, $J_{C\text{-}1,H\text{-}1}$ 157.9 Hz), 99.8 (C-1'', $J_{C\text{-}1'',H\text{-}1''}$ 158.4 Hz), 97.9 (C-1', $J_{C\text{-}1',H\text{-}1'}$ 169.3 Hz), 84.7 (C-3), 83.0 (C-3''), 82.4 (C-2), 78.0 (C-4), 77.5 (C-5'), 76.8 (C-3'), 75.7, 75.0, 74.9, 74.8, 73.6, 72.8 ($PhCH_2$), 75.2 (C-4''), 74.6 (C-4'), 74.4 (C-5), 73.3 (C-2'), 72.8 (C-2''), 71.5 (C-5''), 70.2 (octyl C1), 69.9 (C-6'), 69.4 (C-6''), 66.0 (C-6), 31.8, 29.8, 29.5, 29.3, 26.2, 22.6 (octyl C2-C7), 20.9 (OAc), 14.1 (octyl CH_3); LR MS (ES) calcd. for $C_{91}H_{104}O_{17}Na$ (M + Na) 1492, found *m/z* 1492.

Octyl 3,4,6-tri-O-benzyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**52**)

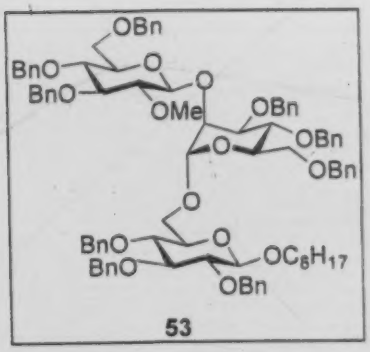


52

Compound **51** (145 mg, 0.10 mmol) was dissolved in dry methanol (3 ml) and a catalytic amount of sodium methoxide was added. The solution was stirred for 24 h until TLC showed complete conversion to the product. The solution was acidified with Amberlite IRC 50 (H^+) resin until acidic to pH paper. After filtration of the resin, the solvent was evaporated. The residue was purified by column chromatography (hexane-EtOAc 6:1) to give **52** (R_f 0.7 hexane-EtOAc 2:1, 143 mg, quant.) as a syrup. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.00 (m, 45 H, Ar-H), 4.98 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.98-4.68 (10×ABq, 10 H, J_{gem} 11.0 Hz., J_{gem} 11.5 Hz, PhC$\underline{H}_2$O), 4.62 (ABq, 1 H, J_{gem} 11.5 Hz, PhC$\underline{H}_2$O), 4.57-4.50 (4×ABq, 4 H, J_{gem} 11.0 Hz, PhC$\underline{H}_2$O), 4.44-4.37 (3×ABq, 3 H, J_{gem} 11.0 Hz, PhC$\underline{H}_2$O), 4.37 (d, 1 H, $J_{1'',2''}$ 7.5 Hz, H-1''), 4.33 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.22 (t, 1 H, $J_{2',3'}$ 3.0 Hz, $J_{2',1'}$ 1.5 Hz, H-2'), 4.01 (t, 1 H, J 9.5 Hz, H-4'), 3.92-3.85 (m, 2 H, H-3', octyl C1-Ha), 3.83 (dd, 1H, J_{gem} 11.0 Hz, $J_{6a,5}$ 4.5 Hz, H-6a), 3.75-3.53 (m, 11 H, H-6a'', H-5', H-6b'', H-5'', H-4'', H-6b, H-3, H-2'', H-3'', H-6a', H-6b'), 3.46-3.35 (m, 4 H, octyl C1-Hb, H-4, H-2, H-5), 1.62-1.49 (m, 2 H, octyl C2-H), 1.37-1.16 (m, 10 H, 5 octyl CH_2), 0.84 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 138.9, 138.7, 138.5, 138.4, 138.2, 138.1, 138.0, 137.7 (quaternary Ph), 128.5, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3 (Ar-H), 103.6 (C-1, $J_{C-1,H-1}$ 158.4 Hz), 101.3 (C-1'', $J_{C-1'',H-1''}$ 157.9 Hz), 98.2 (C-1', $J_{C-1',H-1'}$ 170.0 Hz), 84.8 (C-3), 84.4 (C-3''), 82.4 (C-2), 77.7 (C-4, C-5'), 77.5 (C-3'), 75.7, 75.6, 75.1, 75.0, 74.9, 74.8, 73.5, 73.3, 71.6 ($PhCH_2$), 74.4 (C-4'), 74.2 (C-5), 73.9 (C-2''), 72.4 (C-2'), 71.6 (C-5''), 70.3 (octyl C1), 69.5 (C-6''), 68.7 (C-6'), 66.5 (C-6), 31.9, 29.8, 29.5, 29.4, 26.3, 22.8 (octyl C2-C7), 14.2 (octyl CH_3); LR MS

(ES) calcd. for $C_{89}H_{102}O_{16}Na$ (M + Na) 1450, found *m/z* 1450.

Octyl 3,4,6-tri-O-benzyl-2-O-methyl-β-D-glucopyranosyl-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**53**)

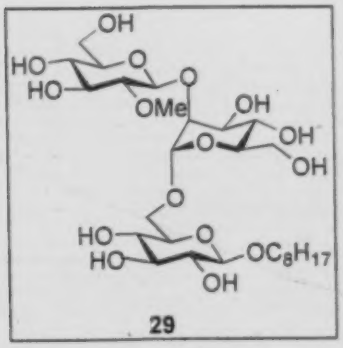


53

Compound **52** (30 mg, 0.02 mmol) was dissolved in dry DMF (1 ml), sodium hydride (15 mg, 80% in oil, 0.50 mmol) was added at 0° C and the solution was stirred for 30 min. Then methyl iodide (0.03 ml, 0.48 mmol) was added dropwise at 0° C and stirring was continued overnight. The reaction flask was diluted with CH_2Cl_2 and the solution was sequentially washed with saturated sodium bicarbonate, water and sodium chloride. Then it was dried over sodium sulfate and the solution was filtered and concentrated. The residue was purified by column chromatography with hexane-EtOAc 4:1 to give **53** (R_f 0.54 hexane-EtOAc 3:1, 29 mg, 99%) as a syrup ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.00 (m, 45 H, Ar-H), 4.99 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.96-4.91 (2×ABq, 3 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.91-4.78 (3×ABq, 3 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.77-4.68 (4×ABq, 4 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.57-4.42 (m, 6 H, PhC$\underline{H_2}$O), 4.40-4.35 (2×ABq, 2 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.36 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.33 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.25 (dd, 1 H, $J_{2',3'}$ 3.0 Hz, $J_{2',1'}$ 1.5 Hz, H-2'), 3.95-3.82 (m, 4 H, H-4', octyl C1-Ha, H-6a, H-3'), 3.74 -3.48 (m, 13 H, , H-5', H-6b, H-6a', H-6b', H-6a'', H-6b'', H-5'', H-3, H-4'', H-3'', OCH_3,), 3.48-3.33 (m, 4 H, octyl C1-Hb, H-5, H-2, H-4), 3.25 (dd, 1 H, $J_{2'',3''}$ 8.5 Hz, $J_{2'',1''}$ 8.0 Hz, H-2''), 1.66-1.54 (m, 2 H, octyl C2-H), 1.37-1.16 (m, 10 H, 5 octyl CH_2), 0.84 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 138.9, 138.8, 138.6, 138.4, 138.2, 138.1, 138.0 (quaternary Ph), 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 127.2 (Ar-H), 103.7 (C-1, $J_{C\text{-}1,H\text{-}1}$ 153.1 Hz), 102.6 (C-1'', $J_{C\text{-}1'',H\text{-}1''}$ 153.1 Hz), 98.3 (C-1', J_{C}-

$J_{1'',H\text{-}1'}$ 170.4 Hz), 85.0 (C-3''), 84.8 (C-3), 83.6 (C-2''), 82.4 (C-2), 77.6 (C-4, C-5'), 77.5 (C-3'), 75.7, 75.4, 75.0, 74.9, 74.8, 73.5, 73.0, 70.1 ($PhCH_2$), 74.7 (C-4''), 74.3 (C-5), 74.1 (C-4'), 73.5 (C-2'), 71.5 (C-5''), 70.4 (octyl C1), 69.7 (C-6'), 69.2 (C-6''), 66.2 (C-6), 60.7 (OCH_3), 31.9, 29.9, 29.5, 29.4, 26.3, 22.8 (octyl C2-C7), 14.2 (octyl CH_3); LR MS (ES) calcd. for $C_{90}H_{104}O_{16}Na$ (M + Na) 1464, found *m/z* 1464.

Octyl 2-O-methyl-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside (**29**)

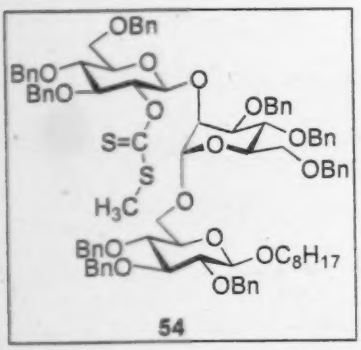


29

Compound **53** (20 mg, 0.01 mmol) was dissolved in dry methanol (2 ml) and 20% palladium hydroxide on carbon (14 mg) was added. The resulting mixture was hydrogenated (1 atm of H_2) for 2 h. Upon completion of the reaction the catalyst was filtered over Celite and the solution was centrifuged to remove the rest of the catalyst. The mixture was concentrated and it was then dissolved in water. The trisaccharide was purified by passing it through a Waters C_{18} Sep-Pak cartridge. The cartridge was previously washed with methanol and then water. The cartridge was washed with water, and the product was eluted with methanol. The methanol eluant was evaporated, the residue redissolved in water, filtered through a 0.22 μM Millex filter and lyophilised to give **29** (6.1 mg, 96%) as a white solid. ^{1}H NMR (D_2O, 500 MHz): δ 5.03 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.57 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.46 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.18 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 1.5 Hz, H-2'), 3.96 (dd, 1 H, J_{gem} 11.5 Hz , $J_{6a,5}$ 5.0 Hz, H-6a), 3.92-3.85 (m, 4 H, H-3', octyl C1-Ha , H-6a'', H-6a'), 3.81 (dd, 1 H, J_{gem} 11.5 Hz , $J_{6b,5}$ 2.0 Hz, H-6b), 3.77-3.65 (m, 5 H, H-5', octyl C1-Hb, H-6b'', H-6b', H-4'), 3.62 (s, 3 H, OCH_3), 3.62-3.57 (m, 1 H, H-5), 3.52-3.38 (m, 5 H, H-3, H-3'', H-4, H-4'', H-5''), 3.26 (ddd, 1 H, $J_{2,1}$ 8.0 Hz, J 7.0 Hz, J 2.5 Hz, H-2),

3.08 (dd, 1 H, $J_{2'',3''}$ 9.0 Hz, $J_{2'',1''}$ 8.0 Hz, H-2''), 1.63 (p, 2 H, J 7.0 Hz, octyl C2-H), 1.39-1.26 (m, 10 H, 5 octyl CH_2), 0.87 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR (D_2O, 125 MHz): δ 103.1 (C-1, $J_{C-1,H-1}$ 160.8 Hz), 101.8 (C-1'', $J_{C-1'',H-1''}$ 162.1 Hz), 98.0 (C-1', $J_{C-1',H-1'}$ 170.9 Hz), 83.2 (C-2''), 77.6 (C-2'), 76.9 (C-3''), 76.6 (C-3), 75.8 (C-5'), 74.9 (C-5), 74.0 (C-2), 73.7 (C-5''), 71.6 (octyl C1), 70.4 (C-3', C-4), 70.3 (C-4''), 68.0 (C-4'), 66.6 (C-6), 61.8 (C-6'), 61.5 (C-6''), 61.3 (OCH_3), 32.0, 29.7, 29.3, 29.3, 26.0, 22.9 (octyl C2-C7), 14.3 (octyl CH_3); HR MS (ES) calcd. for $C_{27}H_{50}O_{16}Na$ (M + Na) 653.2997, found *m/z* 653.3002.

Octyl 3,4,6-tri-O-benzyl-3-O-[(methylthio)thiocarbonyl]-β-D-glucopyranosyl-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside **(54)**

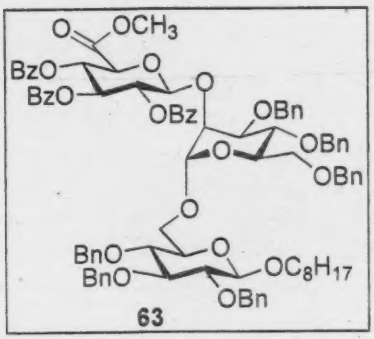


54

To a solution of **52** (103 mg, 0.07 mmol) in dry tetrahydrofuran (1.5 ml) was added sodium hydride (10 mg, 80% in oil, 0.33 mmol), and imidazole (2 mg). The solution was stirred for 1 h and carbon disulfide (68 µl, 1.12 mmol) was added. One hour later, methyl iodide (21 µl, 0.34 mmol) was added and stirring continued overnight. The solution was concentrated to yield a yellow residue which was chromatographed (hexane-EtOAc 10:1) to give **54** (R_f 0.56 in hexane-EtOAc, 106 mg, quant.) as a yellow oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.00 (m, 45 H, Ar-H), 6.12 (dd, 1 H, $J_{2'',3''}$ 9.5 Hz, $J_{2'',1''}$ 8.0 Hz, H-2''), 4.95-4.90 (2×ABq, 2 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.88 (d, 1 H, $J_{1',2'}$ 2.0 Hz, H-1'), 4.85-4.64 (8×ABq, 8 H, J_{gem} 12.0 Hz, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.63 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.57-4.36 (8×ABq, 8 H, J_{gem} 12.0 Hz, , J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.34 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.17 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 2.0 Hz, H-2'), 3.92 (dt, 1 H, J_{gem} 9.5 Hz, J_{vic} 6.5 Hz, octyl C1-Ha), 3.87 (dd, 1 H, J_{gem} 12.0 Hz , $J_{6a,5}$ 4.0 Hz, H-6a), 3.85 (t, 1 H, J 9.0 Hz, H-3''), 3.81 (dd, 1 H,

$J_{3',4'}$ 8.5 Hz , $J_{3',2'}$ 3.0 Hz, H-3'), 3.50-3.39 (m, 3 H, H-4, H-6b', octyl C1-Hb), 3.37 (dd,1 H, $J_{2,3}$ 9.0 Hz, $J_{2,1}$ 8.0 Hz, H-2), 3.33 (ddd, 1 H, $J_{5,4}$ 12.0 Hz , $J_{5,6b}$ 6.0 Hz, $J_{5,6a}$ 4.0 Hz, H-5), 3.75-3.53 (m, 9 H, H-5', H-6a'', H-6b'', H-6b, H-4'', H-5'', H-3, H-6a', H-4'), 2.45 (s, 3 H, SCH_3), 1.66-1.51 (m, 2 H, octyl C2-H), 1.40-1.16 (m, 10 H, 5 octyl CH_2), 0.84 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 215.0 (C=S), 138.7, 138.5, 138.3, 138.1, 138.0, 137.9 (quaternary Ph), 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.5, 127.3 (Ar-H), 103.7 (C-1, $J_{C\text{-}1,H\text{-}1}$ 157.7 Hz), 99.6 (C-1'', $J_{C\text{-}1'',H\text{-}1''}$ 160.2 Hz), 98.2 (C-1', $J_{C\text{-}1',H\text{-}1'}$ 169.8 Hz), 84.7 (C-3), 83.1 (C-3''), 82.4 (C-2), 81.1 (C-2''), 77.8 (C-5'), 77.5 (C-4), 75.7, 75.0, 74.9, 74.8, 74.6, 73.5, 72.8 ($PhCH_2$), 75.1 (C-4''), 74.9 (C-4'), 74.4 (C-5), 73.8 (C-2'), 71.7 (C-5''), 70.3 (octyl C1), 70.2 (C-6'), 69.2 (C-6''), 66.0 (C-6), 60.7 (OCH_3), 31.8, 29.8, 29.5, 29.3, 26.2, 22.6 (octyl C2-C7), 19.2 (SCH_3), 14.2 (octyl CH_3); LR MS (ES) calcd. for $C_{91}H_{104}O_{16}S_2Na$ (M + Na) 1540, found *m/z* 1540.

Octyl (methyl 2,3,4,-tri-O-benzoyl-β-D-glucopyranosyluronate)-(1→2)-3,4,6-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**63**)

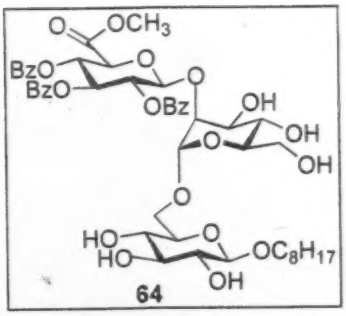


63

A mixture of the alcohol **37** (321 mg, 0.32 mmol), methyl 2,3,4-tri-O-benzoyl-1-O-trichloroacetimidoyl-α-D-glucopyranuronate **60** (303 mg, 0.46 mmol) and powdered 4 Å molecular sieves (0.34 g) in dry dichloromethane (5.8 ml) was stirred for 1 h under argon. A solution of trimethylsilyl triflate in dry toluene (1 mol dm^3, 46 μl, 0.05 mmol) was added at –20° C, and the mixture was stirred for 1 h at the same temperature. A solution of trimethylsilyl triflate in dry toluene (1 mol dm^3, 23 μl, 0.02 mmol) was added again and after stirring for 30 min, the solution was allowed to warm up to room temperature within an hour. It

was then treated with triethylamine (24 μl, 0.17 mmol), filtered, and concentrated. The residue was purified by chromatography hexane-EtOAc 5:1 to give **63** (R_f 0.58 in hexane-EtOAc 2:1, 436 mg, 91%) as a syrup. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.96-7.80 (m, 6 H, "o" Ar-H in OBz), 7.55-7.00 (m, 39 H, Ar-H), 5.89 (t, 1 H, J 9.5 Hz, H-3''), 5.75 (t, 1 H, J 9.5 Hz, H-4''), 5.64 (dd, 1 H, $J_{2'',3''}$ 9.5 Hz, $J_{2'',1''}$ 8.0 Hz, H-2''), 5.10 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.97 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.92 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.91 (d, 1 H, $J_{1',2'}$ 2.0 Hz, H-1'), 4.82-4.74 (2×ABq, 4 H, J_{gem} 12.0 Hz, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.77 (2×ABq, 3 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.62 (ABq, 1 H, J_{gem} 12.0 Hz, PhC$\underline{H_2}$O), 4.54 (d, 1 H, $J_{5'',4''}$ 9.8 Hz, H-5''), 4.40 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.40 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.35 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.26 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 2.0 Hz, H-2'), 4.09 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 6.5 Hz, octyl C1-Ha), 3.88 (dd, 1 H, J_{gem} 12.0 Hz , $J_{6a,5}$ 3.0 Hz, H-6a), 3.84 (dd, 1 H, $J_{3',4'}$ 7.0 Hz, $J_{3',2'}$ 3.5 Hz, H-3'), 3.68-3.58 (m, 8 H, H-4', H-5', OCH_3, H-6b, H-3, octyl C1-Hb), 3.45-3.35 (m, 3 H, H-6a', H-4, H-2), 3.33 (dt, 1 H, J 10.0 Hz, J 2.0 Hz, H-5), 3.22 (dd, 1 H, J_{gem} 11.0 Hz , $J_{6b',5'}$ 5.5 Hz, H-6b'), 1.78-1.69 (m, 2 H, octyl C2-H), 1.44-1.14 (m, 10 H, 5 octyl CH_2), 0.82 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 167.0 (C=O of ester), 165.6, 165.1, 164.8 (C=O), 138.6, 138.5, 138.4, 138.3, 138.0 (quaternary Ph), 133.3, 133.2, 132.9, 129.8, 129.7 ('o' Ar-H in OBz), 129.5, 129.0, 128.9 (quaternary Ph in OBz), 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.2 (Ar-H), 103.9 (C-1, $J_{C\text{-}1,H\text{-}1}$ 158.2 Hz), 99.2 (C-1'', $J_{C\text{-}1'',H\text{-}1''}$ 162.2 Hz), 98.6 (C-1', $J_{C\text{-}1',H\text{-}1'}$ 168.9 Hz), 84.6 (C-3), 82.6 (C-2), 77.3 (C-4), 76.8 (C-3'), 75.7, 75.0, 74.9, 74.5, 72.9, 71.4 ($PhCH_2$), 74.6 (C-4', C-5), 74.3 (C-2'), 72.8 (C-5''), 72.6 (C-3''), 72.1 (C-5'), 71.6 (C-2''), 70.7 (octyl C1), 70.3 (C-4''), 69.8 (C-6'), 66.5 (C-6), 52.7 (OCH_3), 31.8, 29.9, 29.5, 29.3, 26.2, 22.6 (octyl C2-C7), 14.1 (octyl CH_3); LR MS (ES) calcd. for $C_{90}H_{96}O_{20}Na$ (M + Na) 1520, found *m/z* 1520.

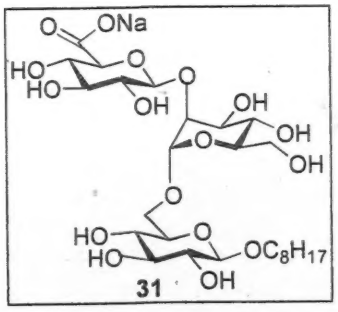
Octyl (methyl 2,3,4,-tri-O-benzoyl-β-D-glucopyranosyluronate)-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside (**64**)



64

A solution of the protected trisaccharide **63** (186 mg, 0.12 mmol) in dry methanol (20 ml) was hydrogenated over 20% palladium hydroxide on carbon (100 mg). After 5 h, the solution was filtered over Celite and the solution was centrifuged to remove traces of the catalyst. The solvent was evaporated and the syrup was dried under vacuum to give **64** (R_f 0.19 in CH_2Cl_2- MeOH 10:1, 115 mg, quant.) ^{1}H NMR (CD_3OD, 500 MHz): δ 7.97-7.80 (3×d, 6 H, 'o' Ar-H of OBz), 7.60-7.30 (m, 9 H, Ar-H), 5.98 (t, 1 H, J 9.5 Hz, H-3''), 5.60 (t, 1 H, J 9.5 Hz, H-4''), 5.50 (dd, 1 H, $J_{2'',3''}$ 9.5 Hz, $J_{2'',1''}$ 8.0 Hz, H-2''), 5.20 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.91 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.64 (d, 1 H, $J_{1'',2''}$ 9.8 Hz, H-5''), 4.26 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.08 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 1.5 Hz, H-2'), 3.94 (dt, 1 H, J_{gem} 9.5 Hz, J_{vic} 7.0 Hz, octyl C1-Ha), 3.82 (dd, 1 H, J_{gem} 11.5 Hz , $J_{6a,5}$ 5.0 Hz, H-6a), 3.72 (dd, 1 H, $J_{3',4'}$ 8.5 Hz , $J_{3',2'}$ 3.5 Hz, H-3'), 3.67-3.56 (m, 6 H, OCH_3, H-6a', H-6b, octyl C1-Hb), 3.51-3.44 (m, 2 H, H-4', H-5'), 3.40-3.32 (m, 4 H, H-4, H-3, H-5, H-6b'), 3.17 (t, 1 H, J 8.0 Hz, H-2), 1.78-1.68 (m, 2 H, octyl C2-H), 1.50-1.18 (m, 10 H, 5 octyl CH_2), 0.84 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR (CD_3OD, 125 MHz): δ 169.4 (C=O of ester), 166.9, 166.8, 166.7 (C=O), 134.9, 134.7 ('o' Ar-H of OBz), 130.8, 130.7, 130.6, 129.7, 129.6 (Ar-H), 130.7, 130.1 (quaternary Ar), 104.7 (C-1, $J_{C\text{-}1,H\text{-}1}$ 157.1 Hz), 100.8 (C-1'', $J_{C\text{-}1'',H\text{-}1''}$ 164.0 Hz), 98.9 (C-1', $J_{C\text{-}1',H\text{-}1'}$ 169.7 Hz), 79.9 (C-2'), 78.2 (C-3), 76.7 (C-5), 75.2 (C-2), 74.7 (C-5'), 73.7 (C-3''), 73.5 (C-5''), 73.0 (C-2''), 71.7 (C-4''), 71.5 (octyl C1), 71.5 (C-3'), 71.0 (C-4), 69.2 (C-4'), 67.2 (C-6), 63.4 (C-6'), 53.4 (OCH_3), 33.0, 31.0, 30.7, 30.5, 27.1, 23.7 (octyl C2-C7), 14.4 (octyl CH_3); HR MS (ES) calcd. for $C_{48}H_{60}O_{20}Na$ (M + Na) 979.3576 , found *m/z* 979.3578.

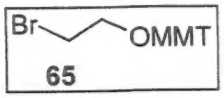
Octyl β-D-glucopyranosyluronic acid-(1→2)-α-D-mannopyranosyl-(1→6)-β-D-glucopyranoside sodium salt (**31**)



31

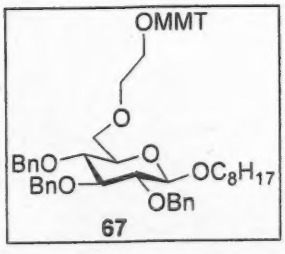
Aq. sodium hydroxide (3 M, 0.6 ml) was added at 0° C to a solution of **64** (30 mg, 0.03 mmol) in methanol and water (5:1, 1.8 ml). The solution was stirred overnight and the pH of the solution was brought to ~ 8 with dil. hydrochloric acid. The mixture was then concentrated. The residue was passed through a column of Sephadex G-10 with water and, after filtration through a 0.22 μM filter, it was freeze dried to give **31** as a white solid (R_f 0.4 in CH_2Cl_2- MeOH-H_2O 7:3:0.5, 17 mg, 89 %) ^{1}H NMR (D_2O, 600 MHz): δ 5.01 (d, 1 H, $J_{1',2'}$ 1.3 Hz, H-1'), 4.54 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.45 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.14 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, $J_{2',1'}$ 1.3 Hz, H-2'), 3.93 (dd, 1 H, J_{gem} 11.5 Hz , $J_{6a,5}$ 5.0 Hz, H-6a), 3.90-3.79 (m, 6 H, H-3', octyl C1-Ha, H-5'', H-6a', H-6b', H-6b), 3.74-3.65 (m, 3 H, H-4'', H-5', octyl C1-Hb), 3.59 (m, 1 H, H-5), 3.57 (t, 1 H, J 9.0 Hz, H-4''), 3.52 (t, 1 H, J 9.0 Hz, H-3''), 3.49-3.44 (m, 2 H, H-3, H-4), 3.39 (dd, 1 H, $J_{2'',3''}$ 9.0 Hz , $J_{2'',1''}$ 8.0 Hz, H-2''), 3.25 (t, 1 H, J 8.0 Hz, H-2), 1.66-1.58 (m, 2 H, octyl C2-H), 1.39-1.23 (m, 10 H, 5 octyl CH_2), 0.86 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR (D_2O, 125 MHz): δ 174.8 (C=O of acid), 103.1 (C-1, $J_{C-1,H-1}$ 161.2 Hz), 102.3 (C-1'', $J_{C-1'',H-1''}$ 162.2 Hz), 98.4 (C-1', $J_{C-1',H-1'}$ 171.5 Hz), 78.3 (C-2'), 76.8 (C-3), 75.9 (C-5'', C-3''), 74.8 (C-5), 73.9 (C-2), 73.4 (C-5'), 73.2 (C-2''), 72.3 (C-4''), 71.6 (octyl C1), 70.5 (C-3'), 70.4 (C-4), 67.5 (C-4'), 66.7 (C-6), 61.1 (C-6'), 31.9, 29.6, 29.2, 25.8, 22.8 (octyl C2-C7), 14.2 (octyl CH_3); HR MS (ES) calcd. for $C_{26}H_{45}O_{17}Na_2$ (M + Na) 675.2452 , found *m/z* 675.2448.

1-[(2-bromoethoxy) diphenyl methyl]-4-methoxy benzene (**65**)



2-Bromoethanol (1.2 ml, 0.02 mol) was dissolved in anhydrous pyridine (25 ml) and chloro-4-methoxy phenyl diphenylmethane (5.35 g, 0.02 mol) was added. The solution was stirred overnight and coevaporated with toluene. The resulting residue was purified by column chromatography (hexane-EtOAc 6:1) to give **65** (R_f 0.7 in hexane –EtOAc 3:1, 7.16 g, 90%) as a cream solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.48-7.42 (m, 4 H, Ar-H), 7.36-7.20 (m, 8 H, Ar-H), 6.86-6.81 (m, 2 H, Ar-H 'o' to OCH_3), 3.79 (s, 3 H, OCH_3), 3.57 (t, 2 H, J 6.0 Hz, CH_2Br), 3.38 (t, 2 H, J 6.0 Hz, OCH_2) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 158.6 (quaternary OCH_3-Ph), 144.3, 135.4 (quaternary Ph), 130.3, 129.2, 128.4, 127.8, 127.7, 127.0 (Ar-H), 113.2, 113.1 (OCH_3-Ar-H), 86.5 (quaternary C), 63.9 (OCH_2), 55.2 (OCH_3), 43.4 (CH_2Br); HR MS (EI) calcd. for $C_{22}H_{21}O_2Br$ 398.0704, found *m/z* 398.0716

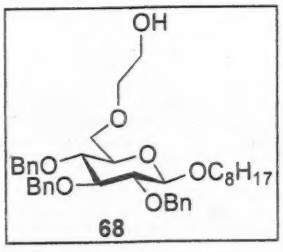
Octyl 2,3,4-tri-O-benzyl-6-O-[(4-methoxyphenyldiphenylmethyl)-2-hydroxyethyl]-β-D-glucopyranoside (**67**)



Compound **34** (0.5 g, 0.890 mmol) was dissolved in dry DMF (4 ml) and sodium hydride (80% in oil, 27 mg, 0.90 mmol) was added at 0° C under argon. After stirring for 30 min, 1-[(2-bromoethoxy) diphenyl methyl]-4-methoxy benzene **65** (0.35 g, 0.895 mmol) was added slowly at 0° C. The reaction was monitored carefully until all the alcohol was consumed. Methanol was added and the pH of the solution was checked to make sure it was basic. After evaporation of the solvent, the residue was dissolved in ethyl acetate and washed successively with water, brine and dried over sodium sulfate. The solution was filtered, concentrated and the residue was purified by column chromatography (hexane-ethyl acetate 7:1) to afford **67** (R_f 0.65 hexane-EtOAc 3:1, 0.63 g, 80%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ

7.57-7.39 (m, 4 H, MMT Ar-H), 7.35-7.14 (m, 23 H, Ar-H, MMT Ar-H), 6.85-6.75 (m, 2 H, MMT Ar-H), 4.93 (ABq, 2 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.82 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.79 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.70 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.67 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.37 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.91 (dt, 1 H, J_{gem} 9.5 Hz, J_{vic} 6.5 Hz, octyl C1-Ha), 3.80-3.61 (m, 9 H, H-6a, H-6b, OCH_3, ethyl C1-Ha, ethyl C1-Hb, H-4, H-3), 3.50-3.40 (m, 3 H, octyl C1-Hb, H-2, H-5), 3.29-3.23 (m, 1 H, ethyl C2-Ha), 3.22-3.16 (m, 1 H, ethyl C2-Hb), 1.66-1.55 (m, 2 H, octyl C2-H), 1.40-1.15 (m, 10 H, 5 octyl CH_2), 0.85 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 158.4, 144.6, 136.0, 135.9 (quaternary Ph of MMT), 138.8, 138.6, 138.3 (quaternary Ph), 130.5, 130.3 (Ar-H MMT), 128.6, 128.5, 128.4, 128.3, 128.1, 128.0, 127.8, 127.7, 127.6, 127.5 (Ar-H), 126.7, 113.0 (Ar-H MMT), 103.7 (C-1), 86.2 (quaternary C of MMT), 84.7 (C-3), 82.3 (C-2), 77.9 (C-4), 75.6, 75.0, 74.8 ($PhCH_2$), 75.0 (C-5), 71.2 (ethyl C1), 70.1 (C-6), 70.1 (octyl C1), 63.4 (ethyl C2), 55.1 (OCH_3), 31.8, 29.8, 29.4, 29.2, 26.1, 22.6 (octyl C2-C7), 14.1 (octyl CH_3); HR MS (ES) calcd. for $C_{57}H_{66}O_8Na$ (M + Na) 901.4655, found *m/z* 901.4656

Octyl 2,3,4-tri-O-benzyl-6-O-(2-hydroxylethyl)-β-D-glucopyranoside (**68**)

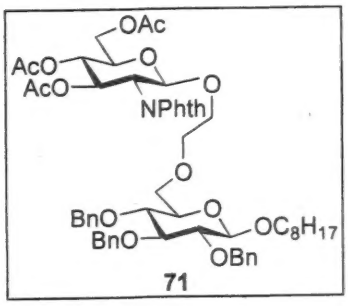


68

Compound **67** (0.41 g, 0.47 mmol) was dissolved in 80% acetic acid (30 ml) and kept for 20 h at room temperature. The mixture was concentrated and the residual water was removed by coevaporation with toluene. The residue was then subjected to gradient column chromatography (hexane-EtOAc 7:1→3:1) to give **68** (R_f 0.37 hexane-EtOAc 3:1, 0.25 g, 90 %) as a syrup. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.15 (m, 15 H, Ar-H), 4.93 (2×ABq, 2 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.85 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.78 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.70 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.60 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.37 (d, 1 H, $J_{1,2}$

7.8 Hz, H-1), 3.91 (dt, 1 H, J_{gem} 9.5 Hz, J_{vic} 6.5 Hz, octyl C1-Ha), 3.76-3.66 (m, 4 H, H-6a, H-6b, ethyl C2-Ha, ethyl C2-Hb), 3.66-3.47 (m, 5 H, H-3, ethyl C1-Ha, ethyl C1-Hb, H-4, octyl C1-Hb), 3.44-3.39 (m, 2 H, H-5, H-2), 2.23(t, 1 H, J 6.0 Hz, O-H), 1.68-1.59 (m, 2 H, octyl C2-H), 1.42-1.20 (m, 10 H, 5 octyl CH_2), 0.86 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 138.6, 138.5, 138.1 (quaternary Ph), 128.4, 128.3, 128.1, 127.9, 127.8, 127.6 (Ar-H), 103.7 (C-1), 84.6 (C-3), 82.3 (C-2), 77.8 (C-4), 75.6, 75.0, 74.8 ($PhCH_2$), 74.7 (C-5), 72.8 (ethyl C1), 70.3 (octyl C1), 70.0 (C-6), 61.8 (ethyl C2), 31.8, 29.8, 29.4, 29.2, 26.1, 22.6 (octyl C2-C7), 14.1 (octyl CH_3); HR MS (ES) calcd. for $C_{37}H_{50}O_7Na$ (M + Na) 629.3454, found *m/z* 629.3455.

Octyl 2,3,4-tri-O-benzyl-6-O-[(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-hydroxyethyl]-β-D-glucopyranoside (**71**)

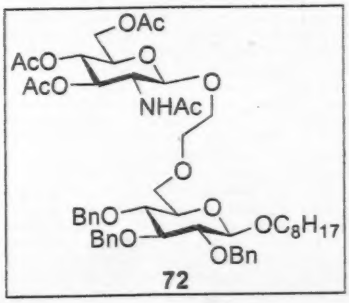


71

Alcohol **68** (180 mg, 0.30 mmol) was dissolved in dry dichloromethane (4.5 ml). Silver trifluoromethanesulfonate (764 mg, 2.97 mmol), sym collidine (0.39 ml, 2.93 mmol), and 4 Å molecular sieves (0.6 g) were added and the mixture was cooled to -50° C. A solution of 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide **66** (148 mg, 0.30 mmol) in CH_2Cl_2 (3 ml) was added and after 15 min at -50° C, the mixture was allowed to warm up to room temperature. The mixture was cooled to -50° C again and more **66** (147 mg, 0.30 mmol) in CH_2Cl_2 (3 ml) was added. Ater stirring for 15 min the solution was allowed to warm up to room temperature and stirred overnight. The mixture was diluted with CH_2Cl_2 (50 ml) and filtered through Celite. The filtrate was washed sequentially with ice-water, cold M HCl, sat. sodium hydrogen carbonate, and finally water. After solvent evaporation the residue was purified by column chromatography (hexane-EtOAc 3:1) to yield **71** (R_f 0.72

hexane-EtOAc 1:1, 298 mg, 97%) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.78-7.71 (bm, 2 H, phthalimido Ar-H), 7.63-7.60 (m, 2 H, phthalimido Ar-H), 7.34-7.17 (m, 15 H, Ar-H), 5.76 (dd, 1 H, $J_{3',2'}$ 10.5 Hz, $J_{3',4'}$ 9.5 Hz, H-3'), 5.42 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 5.13 (t, 1 H, J 9.5 Hz, H-4'), 4.89 (ABq, 2 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.74 (ABq, 2 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.67 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.47 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H_2}$O), 4.32-4.27 (m, 2 H, H-6a', H-2'), 4.27 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.14 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6b',5'}$ 2.0 Hz, H-6b'), 3.91-3.81 (m, 3 H, ethyl C2-Ha, octyl C1-Ha, H-5'), 3.66 (m, 1 H, ethyl C2-Ha), 3.57-3.48 (m, 4 H, H-3, ethyl C1-Ha, ethyl C1-Hb, H-6a), 3.47-3.41 (m, 2 H, octyl C1-Hb, H-6b), 3.39 (t, 1 H, J 9.5 Hz, H-4), 3.32 (dd, 1 H, $J_{2,3}$ 9.0 Hz, $J_{2,1}$ 8.0 Hz, H-2), 3.19 (m, 1 H, H-5), 2.07, 2.01, 1.83 (s, each 3 H, OAc), 1.65-1.54 (m, 2 H, octyl C2-H), 1.41-1.18 (m, 10 H, 5 octyl CH_2), 0.85 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.7, 170.1, 169.4 (C=O), 138.6, 138.5, 138.2 (quaternary Ph), 134.1 (phthalimido Ar-H), 131.4 (quaternary phthalimido Ph), 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5 (Ar-H), 123.5 (phthalimido Ar-H), 103.6 (C-1, $J_{C-1,H-1}$ 159.1 Hz), 98.0 (C-1', $J_{C-1',H-1'}$ 164.6 Hz) 84.6 (C-3), 82.2 (C-2), 77.8 (C-4), 75.6, 74.7 ($PhCH_2$), 74.8 (C-5), 71.8 (C-5'), 70.8 (C-3'), 70.4 (ethyl C1), 70.1 (C-6), 70.0 (octyl C1), 69.0 (C-4'), 68.8 (ethyl C2), 62.0 (C-6'), 31.8, 29.7, 29.4, 29.2, 26.2, 22.6 (octyl C2-C7), 14.1 (octyl CH_3); HR MS (ES) calcd. for $C_{57}H_{69}NO_{16}Na$ (M + Na) 1046.4514, found *m/z* 1046.4514.

Octyl 2,3,4-tri-O-benzyl-6-O-[(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-2-hydroxyethoxy]-β-D-glucopyranoside (**72**)

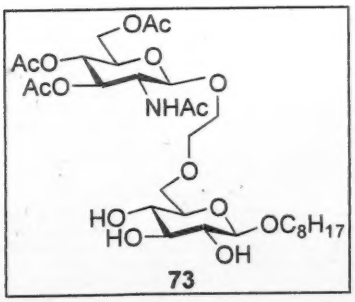


72

Compound **71** (298 mg, 0.29 mmol) in butanol (65 ml) was added to ethylenediamine (12 ml, 0.18 mol) under argon. The solution was stirred for 20 h at 90° C. Reaction was stopped when all starting material was

converted to the product (R_f 0.05 CH_2Cl_2-MeOH 10:1, ninhydrin positive). The mixture was concentrated to dryness and coevaporated twice with toluene. The yellow syrup was acetylated with anhydrous acetic anhydride (1 ml) and pyridine (1 ml) and stirring was continued overnight. After concentration of the mixture, the residue was coevaporated thrice with toluene. Dichloromethane (30 ml) was added to the solution and it was washed sequentially with water, brine and dried over sodium sulfate. The solvent was evaporated and the residue was purified by gradient column chromatography (hexane-EtOAc 1:1→1:2) to give **72** (R_f 0.70 CH_2Cl_2-MeOH, 10:1, 258 mg, 95%) as a syrup. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.40-7.20 (m, 15 H, Ar-H), 5.85 (d, 1 H, $J_{N\text{-}H,H\text{-}2}$ 9.0 Hz, N-H), 5.17 (dd, 1 H, $J_{3',4'}$ 9.5 Hz, $J_{3',2'}$ 9.0 Hz, H-3'), 5.02 (t, 1 H, J 9.5 Hz, H-4'), 4.97 (ABq, 1 H, J_{gem} 11.2 Hz, PhC$\underline{H}_2$O), 4.92 (Abq, 1 H, J_{gem} 11.2 Hz, PhC$\underline{H}_2$O), 4.84 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H}_2$O), 4.77 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H}_2$O), 4.76 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H}_2$O), 4.71 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.61 (ABq, 1 H, J_{gem} 11.0 Hz, PhC$\underline{H}_2$O), 4.37 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.27 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6a',5'}$ 4.5 Hz, H-6a'), 4.11 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6b',5'}$ 2.3 Hz, H-6b'), 3.93-3.83 (m, 3 H, H-2', ethyl C2-Ha, octyl C1-Ha), 3.71-3.57 (m, 7 H, H-5', H-3, H-4, H-6a, H-6b, ethyl C1-Ha, ethyl C2-Hb), 3.53-3.44 (m, 2 H, octyl C1-Hb, ethyl C1-Hb), 3.44-3.36 (m, 2 H, H-2, H-5), 2.06, 1.98, 1.97 (s, each 3 H, OAc), 1.89 (s, 3 H, NHAc), 1.65-1.53 (m, 2 H, octyl C2-H), 1.37-1.18 (m, 10 H, 5 octyl CH_2), 0.85 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.7, 170.3, 170.1 (C=O), 169.4 (C=O, NHAc), 138.5, 138.5, 138.2 (quaternary Ph), 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6 (Ar-H), 104.1 (C-1, $J_{C\text{-}1,H\text{-}1}$ 159.0 Hz), 101.7 (C-1', $J_{C\text{-}1',H\text{-}1'}$ 162.8 Hz) 84.6 (C-3), 82.2 (C-2), 77.2 (C-4), 75.7, 74.9, 74.8 ($PhCH_2$), 74.7 (C-5), 72.6 (C-3'), 71.7 (C-5'), 71.4 (ethyl C1), 70.8 (octyl C1), 69.6 (C-6), 69.0 (C-4'), 68.7 (ethyl C2), 62.1 (C-6'), 54.2 (C-2'), 31.8, 29.7, 29.4, 29.2, 26.1, 22.6 (octyl C2-C7), 23.2 (CH_3 NHAc), 20.7, 20.6 (OAc), 14.0 (octyl CH_3); HR MS (ES) calcd. for $C_{51}H_{69}NO_{15}Na$ (M + Na) 958.4565, found *m/z* 958.4564.

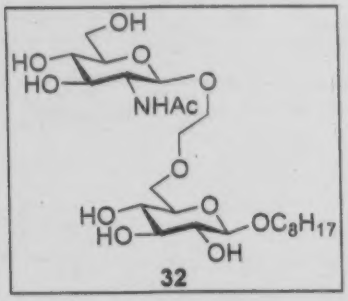
Octyl 6-O-[(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)2-hydroxyethyl]-β-D-glucopyranoside (**73**)



73

Compound **72** (170 mg, 0.182 mmol) was dissolved in dry methanol (10 ml) and it was hydrogenated over 20% palladium hydroxide on carbon (75 mg) for 3 h. The mixture was filtered over Celite, and centrifuged to remove traces of catalyst. The solvent was then evaporated and the residue was dried under vacuum overnight to give **73** (R_f 0.36 CH_2Cl_2-MeOH 10:1, 114 mg, 95 %) as a glass. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.00 (d, 1 H, $J_{N\text{-}H,H\text{-}2}$ 9.0 Hz, N-H), 5.21 (dd, 1 H, $J_{3',4'}$ 10.5 Hz, $J_{3',2'}$ 9.5 Hz, H-3'), 5.04 (dd, 1 H, $J_{4',3'}$ 10.5 Hz, $J_{4',5'}$ 9.5 Hz, H-4'), 4.74 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.25 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.23 (dd, 1 H, J_{gem} 12.5 Hz, $J_{6a',5'}$ 4.5 Hz, H-6a'), 4.13 (dd, 1 H, J_{gem} 12.5 Hz, $J_{6b',5'}$ 2.5 Hz, H-6b'), 3.96-3.83 (m, 3 H, ethyl C2-Ha, H-2', octyl C1-Ha), 3.81 (dd, 1 H, J_{gem} 10.0 Hz, $J_{6a,5}$ 3.7 Hz, H-6a), 3.75-3.61 (m, 4 H, ethyl C2-Hb, H-5', H-6b, H-4), 3.61-3.51 (m, 3 H, ethyl C1-Ha, ethyl C1-Hb, H-3), 3.48 (dt, 1 H, J_{gem} 9.5 Hz, J_{vic} 7.0 Hz, octyl C1-Hb), 3.42-3.35 (m, 2 H, H-5, H-2), 2.50 (bs, 1 H, O-H), 2.07, 2.01, 2.00 (s, each 3 H, OAc), 1.96 (s, 3 H, NHAc), 1.59 (p, 2 H, J_{vic} 7.0 Hz, octyl C2-H), 1.38-1.20 (m, 10 H, 5 octyl CH_2), 0.86 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.9, 170.8 (C=O), 169.3 (C=O, NHAc), 102.8 (C-1, $J_{C\text{-}1,H\text{-}1}$ 156.2 Hz), 101.0 (C-1', $J_{C\text{-}1',H\text{-}1'}$ 162.6 Hz), 76.2 (C-3), 74.2 (C-5), 73.7 (C-2), 72.3 (C-3'), 71.9 (C-4), 71.6 (C-5'), 71.2 (C-6), 71.1 (ethyl C1), 70.4 (octyl C1), 68.7 (C-4'), 68.5 (ethyl C2), 62.1 (C-6'), 54.6 (C-2'), 31.8, 29.6, 29.4, 29.2, 25.9, 22.6 (octyl C2-C7), 23.3 (CH_3 NHAc), 20.8, 20.7, 20.6 (OAc), 14.1 (octyl CH_3); HR MS (ES) calcd. for $C_{30}H_{51}NO_{15}Na$ (M + Na) 688.3156, found *m/z* 688.3155.

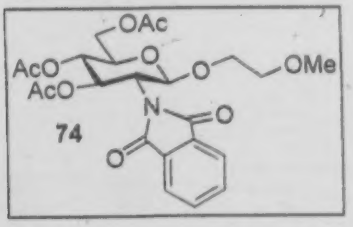
Octyl 6-O-[(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-hydroxyethyl-]-β-D-glucopyranoside (**32**)



32

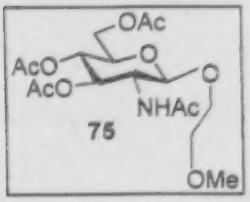
Compound **73** (115 mg, 0.17 mmol) was dissolved in dry methanol (8 ml) and a catalytic amount of sodium methoxide was added at 0° C. The reaction was stirred for 4 h and it was then neutralised with Amberlite IRC 50 (H+). After filtration of the resin the solution was evaporated. It was dissolved in water and passed through a Waters C_{18} Sep-Pak cartridge. It was eluted with methanol and after evaporation of the methanol, water was added and it was filtered through a 0.22 μM millipore filter. After lyophilisation, **32** (R_f 0.70 CH_2Cl_2-MeOH-H_2O 10:6:1, 92 mg, quant.) was obtained as a white solid. ^{1}H NMR (D_2O, 500 MHz): δ 4.57 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.44 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.01 (ddd, 1 H, J_{gem} 11.5 Hz , J 6.0 Hz, J 3.0 Hz, ethyl C2-Ha), 3.95-3.85 (m, 3 H, H-6a', octyl C1-Ha, H-6a), 3.82-3.64 (m, 7 H, ethyl C2-Hb, ethyl C1-Ha, ethyl C1-Hb, H-6b', H-2', octyl C1-Hb, H-6b), 3.57-3.51 (m, 2 H, H-3', H-5), 3.50-3.43 (m, 3 H, H-3, H-5', H-4'), 3.39 (t, 1 H, J 9.5 Hz, H-4), 3.25 (dd, 1 H, $J_{2,3}$ 9.0 Hz, $J_{2,1}$ 8.0 Hz, H-2), 2.05 (s, 3 H, NHAc), 1.62 (p, 2 H, J_{vic} 7.0 Hz, octyl C2-H), 1.39-1.24 (m, 10 H, 5 octyl CH_2), 0.86 (t, 3 H, J 7.0 Hz, octyl CH_3) ^{13}C NMR (D_2O, 125 MHz): δ 175.3 (C=O), 103.1 (C-1, $J_{C-1,H-1}$ 159.9 Hz), 102.0 (C-1', $J_{C-1',H-1'}$ 160.9 Hz), 76.7 (C-5'), 76.6 (C-3), 75.7 (C-3'), 74.7 (C-5), 73.9 (C-2), 71.6 (C-6), 71.2 (ethyl C1), 70.7 (C-4'), 70.7 (octyl C1), 70.6 (C-4), 69.8 (ethyl C2), 61.6 (C-6'), 56.4 (C-2'), 31.9, 29.6, 29.2, 25.8, 22.8 (octyl C2-C7), 23.1 (CH_3 NHAc), 14.2 (octyl CH_3); HR MS (ES) calcd. for $C_{24}H_{45}NO_{12}Na$ (M + Na) 562.2839, found *m/z* 562.2842.

(2-methoxyethyl) 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (74)



Silver trifluoromethanesulfonate (0.55 g, 2.14 mmol), 1,1,3,3-tetramethyl urea (0.18 ml, 1.56 mmol), 2-methoxy ethanol (previously dried over molecular sieves, 0.23 ml, 2.92 mmol) and 4 Å molecular sieves (0.5 g) were stirred in CH_2Cl_2 under nitrogen. The temperature was reduced to –30° C and the bromide **66** (0.97 g, 1.95 mmol) was added. The temperature was allowed to rise to ambient and stirring was continued overnight. The mixture was filtered over Celite and after evaporation of the solvent, the residue was purified by column chromatography (hexane-EtOAc 2:1) to give **74** (R_f 0.44 pet. ether-EtOAc 6:4, 0.99 g, 94%) as white solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.86 (dd, 2 H, J 5.5 Hz, J 3.0 Hz, phthalimido Ar-H), 7.73 (dd, 2 H, J 5.5 Hz, J 3.0 Hz, phthalimido Ar-H), 5.82 (dd, 1 H, $J_{3,2}$ 10.8 Hz, $J_{3,4}$ 9.0 Hz, H-3), 5.43 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 5.17 (dd, 1 H, $J_{4,5}$ 10.1 Hz, $J_{4,3}$ 9.0 Hz, H-4), 4.37-4.29 (m, 2 H, H-2, H-6a), 4.18 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6b,5}$ 2.0 Hz, H-6b), 3.95-3.84 (m, 2 H, ethyl C1-Ha, H-5), 3.68 (ddd, 1 H, J_{gem} 11.5 Hz, J 7.0 Hz, J 3.7 Hz, ethyl C1-Hb), 3.40-3.33 (m, 2 H, ethyl C2-Ha, ethyl C2-Hb), 3.02 (s, 3 H, OCH_3), 2.11, 2.03, 1.87 (s, each 3 H, OAc) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.7, 170.1, 169.4 (C=O), 134.1, 123.5 (Ar-H), 131.5 (quaternary Ph), 98.5 (C-1, $J_{C-1,H-1}$ 165.9 Hz), 71.8 (C-5), 71.3 (ethyl C2), 70.7 (C-3), 69.2 (ethyl C1), 69.0 (C-4), 62.1 (C-6), 58.6 (OCH_3), 54.6 (C-2), 20.7, 20.6, 20.4 (OAc); HR MS (ES) calcd. for $C_{23}H_{27}NO_{11}Na$ (M + Na) 516.1482, found *m/z* 516.1484.

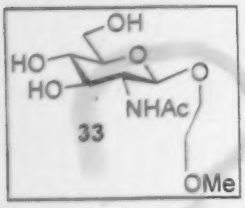
(2-methoxyethyl) 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (**75**)



75

Compound **74** (386 mg, 0.78 mmol) in butanol (80 ml) was added to ethylenediamine (30 ml, 0.45 mol) under argon. The solution was stirred for 20 h at 90° C. Reaction was stopped when all starting material was converted to the product (R_f 0.17 CH_2Cl_2-MeOH 10:1, ninhydrin positive). The mixture was concentrated and the residue was coevaporated twice with toluene. The yellow syrup was acetylated with anhydrous acetic anhydride (3 ml) and pyridine (2.6 ml) and stirring was continued overnight. The solution was coevaporated thrice with toluene. Dichloromethane (30 ml) was added to the solution and it was washed sequentially with water, brine and dried over sodium sulfate. The mixture was filtered and after evaporation of the solvent, the residue was purified by column chromatography (CH_2Cl_2-MeOH 20:1) to give **75** (R_f 0.49 CH_2Cl_2-MeOH 10:1, 300 mg, 95%) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.62 (bs, 1 H, N-H), 5.25 (dd, 1 H, $J_{3,2}$ 10.5 Hz, $J_{3,4}$ 9.5 Hz, H-3), 5.03 (dd, 1 H, $J_{4,5}$ 10.0 Hz, $J_{4,3}$ 9.5 Hz, H-4), 4.74 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.22 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6a,5}$ 4.7 Hz, H-6a), 4.10 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6b,5}$ 2.5 Hz, H-6b), 3.91 (ddd, 1 H, J_{ggm} 11.5 Hz, J 4.5 Hz, J 3.0 Hz, ethyl C1-Ha), 3.83 (dt, 1 H, $J_{2,3}$ 10.5 Hz, $J_{2,1}$ 8.5 Hz, H-2), 3.74-3.65 (m, 2 H, ethyl C1-Hb, H-5), 3.56-3.46 (m, 2 H , ethyl C2-Ha, ethyl C2-Hb), 3.34 (s, 3 H, OCH_3), 2.05, 2.00, 1.99, 1.91 (s, each 3 H, NHAc) ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.8, 170.6, 170.2, 169.3 (C=O), 100.9 (C-1), 72.5 (C-3), 71.9 (ethyl C2, C-5), 68.6 (C-4, ethyl C1), 62.1 (C-6), 59.0 (OCH_3), 54.7 (C-2), 23.3, 20.7, 20.6 (3×OAc, NHAc); HR MS (ES) calcd. for $C_{23}H_{27}NO_{11}Na$ (M + Na) 428.1533, found *m/z* 428.1532.

(2-methoxyethyl) 2-acetamido-2-deoxy-β-D-glucopyranoside (**33**)



Compound **75** (222 mg, 0.55 mmol) was dissolved in methanol (4 ml) and a catalytic amount of sodium methoxide was added. The solution was stirred for 4 h and after solvent evaporation, **33** (R_f 0.17 CH_2Cl_2-MeOH 10:1, 153 mg, quant.) was obtained as a white solid. ^{1}H NMR (CD_3OD, 500 MHz): δ 4.45 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 3.94 (ddd, 1 H, J_{gem} 11.5 Hz, J 5.0 Hz, J 3.5 Hz, ethyl C1-Ha), 3.86 (dd, 1 H, J_{gem} 12.0 Hz, $J_{6a,5}$ 2.0 Hz, H-6a), 3.69-3.64 (m, 2 H, H-6b, ethyl C1-Hb), 3.62 (dd, 1 H, $J_{2,1}$ 8.5 Hz, $J_{2,3}$ 10.0 Hz, H-2), 3.54-3.51 (m, 2 H, ethyl C2-Ha, ethyl C2-Hb), 3.45 (dd, 1 H, $J_{3,4}$ 8.5 Hz, $J_{3,2}$ 10.0 Hz, H-3), 3.34 (s, 3 H, OCH_3), 3.30 (m, 1 H, H-4), 3.25 (ddd, 1 H, $J_{5,4}$ 9.5 Hz, $J_{5,6b}$ 5.0 Hz, $J_{5,6a}$ 2.0 Hz, H-5), 1.96 (s, each 3 H, NHAc) ^{13}C NMR (CD_3OD, 125 MHz): δ 173.8 (C=O), 102.7 (C-1, $J_{C-1,H-1}$ 160.1 Hz), 78.0 (C-5), 76.2 (C-3), 72.9 (ethyl C2), 72.2 (C-4), 69.6 (ethyl C1), 62.8 (C-6), 59.2 (OCH_3), 57.4 (C-2), 23.0 (NHAc); HR MS (ES) calcd. for $C_{11}H_{21}NO_7Na$ (M + Na) 302.1216, found *m/z* 302.1216.

Chapter 6

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Chapter 7

Appendix

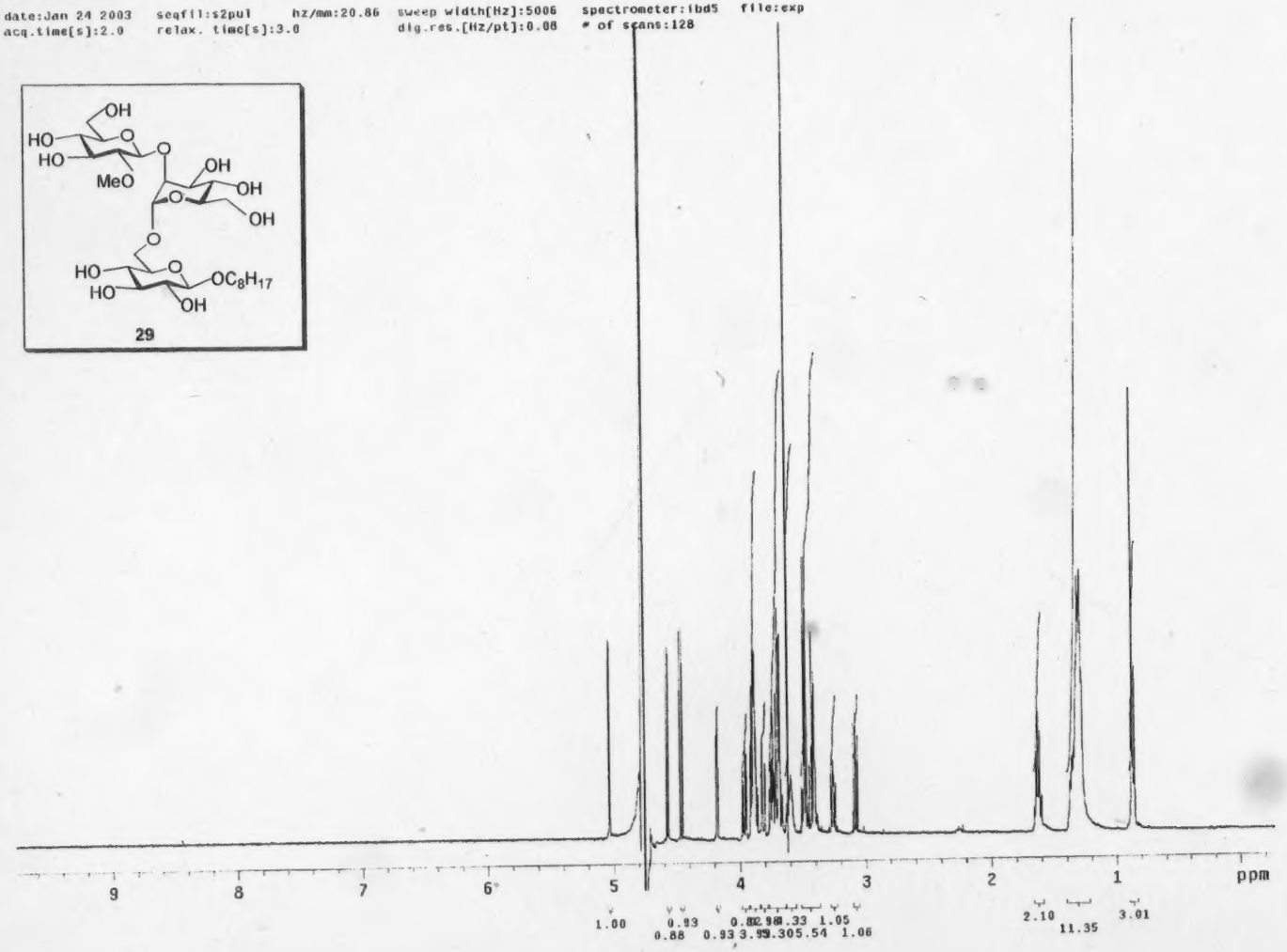
7.1 ^{1}H NMR and ^{13}C NMR Spectra of Final Compounds

500 MHz 1D in D2O (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 27.2 C -> actual temp = 27.0 C, sw500 probe

Pulse Sequence: s2pul

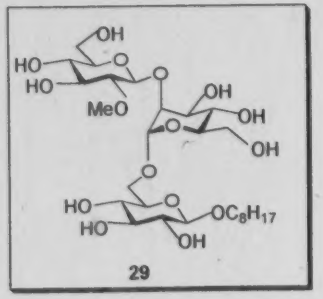
date:Jan 24 2003 seqfil:s2pul hz/mm:20.86 sweep width[Hz]:5006 spectrometer:ibd5 file:exp
acq.time[s]:2.0 relax. time[s]:3.0 dig.res.[Hz/pt]:0.08 # of scans:128

29



125 MHz APT in D2O (ref. to 1% acetone @ 31.07 ppm), temp 27.2 C -> actual temp = 27.0 C, sw probe
CH, CH3 positive; C, CH2 negative
date:Jan 25 2003 seqfil:apt hz/mm:104.17 sweep width[Hz]:25000 spectrometer:ibd5 file:exp
acq.time[s]:2.0 relax. time[s]:0.5 dig.res.[Hz/pt]:0.38 # of scans:27392

Pulse Sequence: apt



29

103.111
101.787
98.038
83.241
77.552
76.867
76.568
75.813
74.872
73.965
73.666
71.620
70.417
70.382
70.344
68.036
66.631
61.782
61.461
61.330
31.881
29.695
29.314
29.262
25.952
22.831
14.346

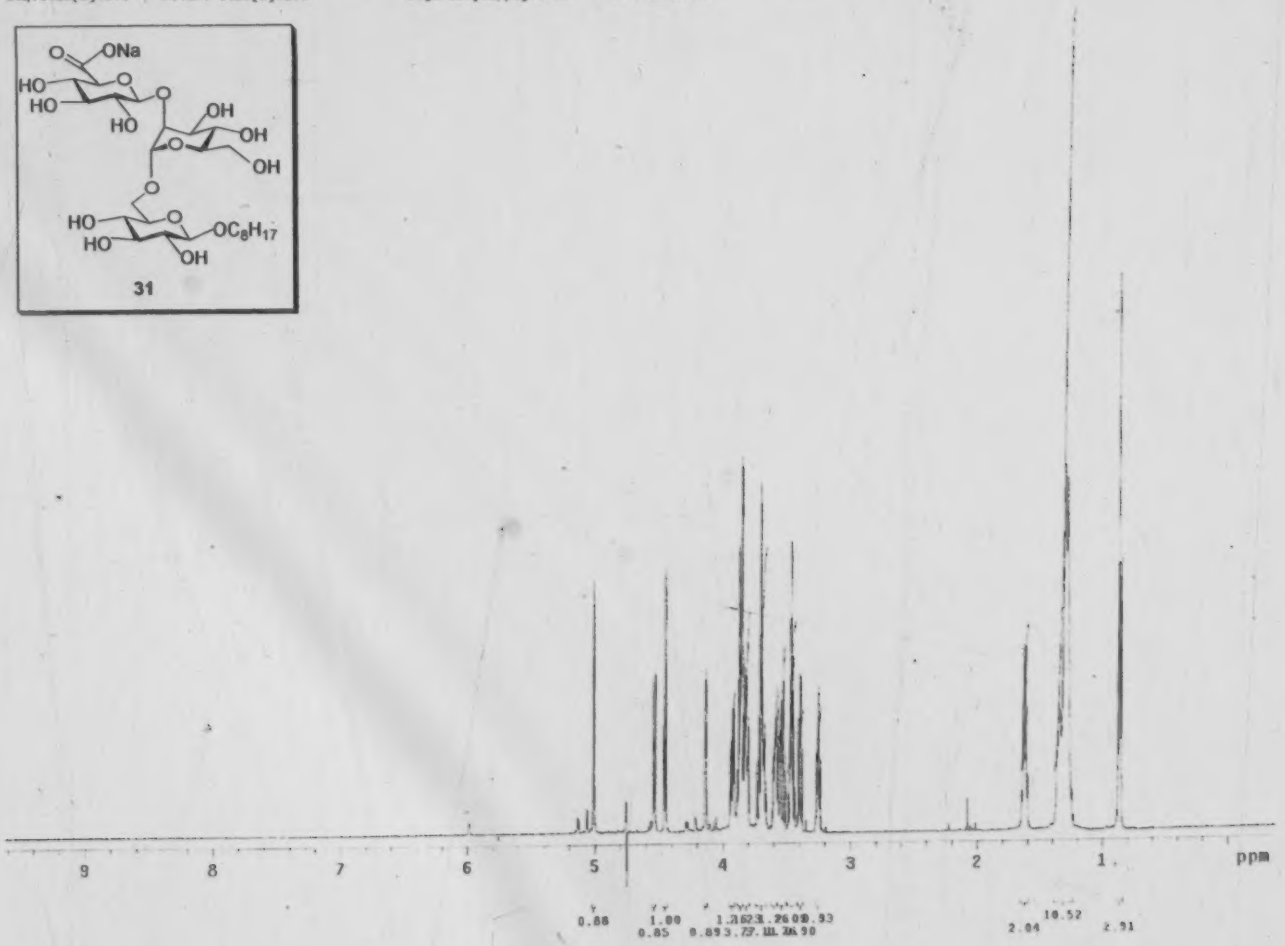
180 160 140 120 100 80 60 40 20 ppm

600 MHz IB in D20 (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 28.0 C -> actual temp = 27.0 C, 1d600 probe

Pulse Sequence: s2pul

date:Apr 21 2003 seqfil:s2pul hz/mm:25.00 sweep width[Hz]:6001 spectrometer:1600 file:exp
acq.time[s]:2.0 relax. time[s]:3.0 dig.res.[Hz/pt]:0.09 # of scans: 44

31



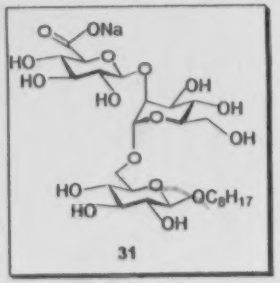
DG-3-135-125 MHz APT in D2O (ref. to 1% acetone @ 31.07 ppm), temp 27.2 C -> actual temp = 27.0 C, sw probe

Pulse Sequence: apt

C & CH2 opposite side of CH & CH3

date:Apr 19 2003 seqfil:apt hz/mm:130.41 sweep width[Hz]:31299 spectrometer:ibd5 file:/mnt/d600/olenmrdata/dristee/DG-3-135-APT-500.fid

acq.time[s]:2.0 relax. time[s]:0.1 dig.res.[Hz/pt]:0.48 # of scans:33324



31

174.778

103.110
102.328
98.300
78.302
76.826
75.423
74.756
73.313
73.422
73.165
72.305
71.589
70.528
70.486
70.425
70.366
70.333
67.512
66.686
61.149

31.880
29.585
29.204
29.151
25.837
22.810
14.217

220 200 180 160 140 120 100 80 60 40 20 ppm

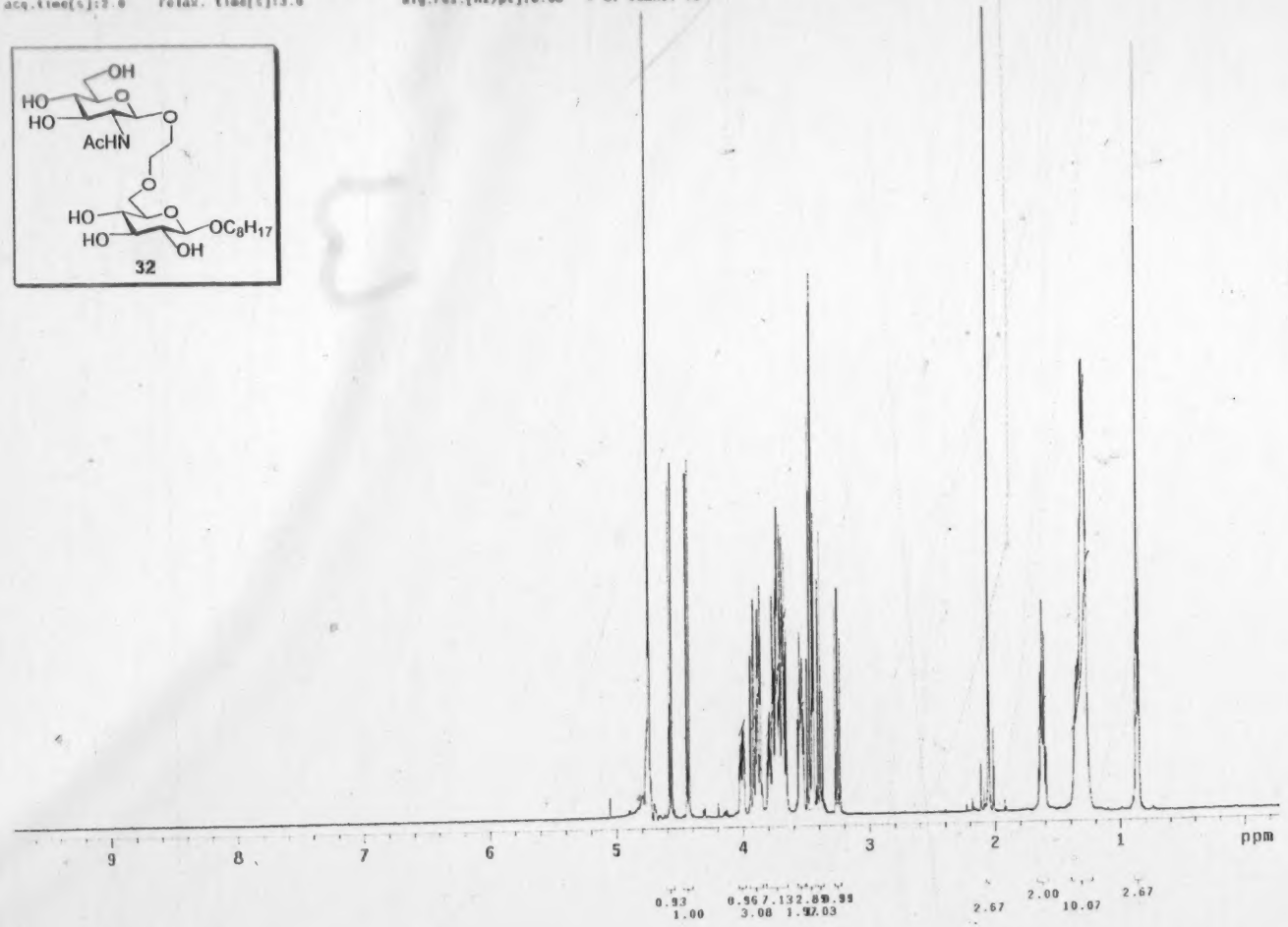
111

DG-3-77-500 MHz 1D in D20 (ref. to 0.1 % ext. acetone @ 2.225 ppm), temp 27.2 C -> actual temp = 27.0 C, sw500 probe

Pulse Sequence: s2pul

date:Apr 15 2003 seqfil:s2pul hz/mm:20.86 sweep width[Hz]:5006 spectrometer:ibd5 file:exp
acq.time[s]:2.0 relax. time[s]:3.0 dig.res.[Hz/pt]:0.08 # of scans: 76

32

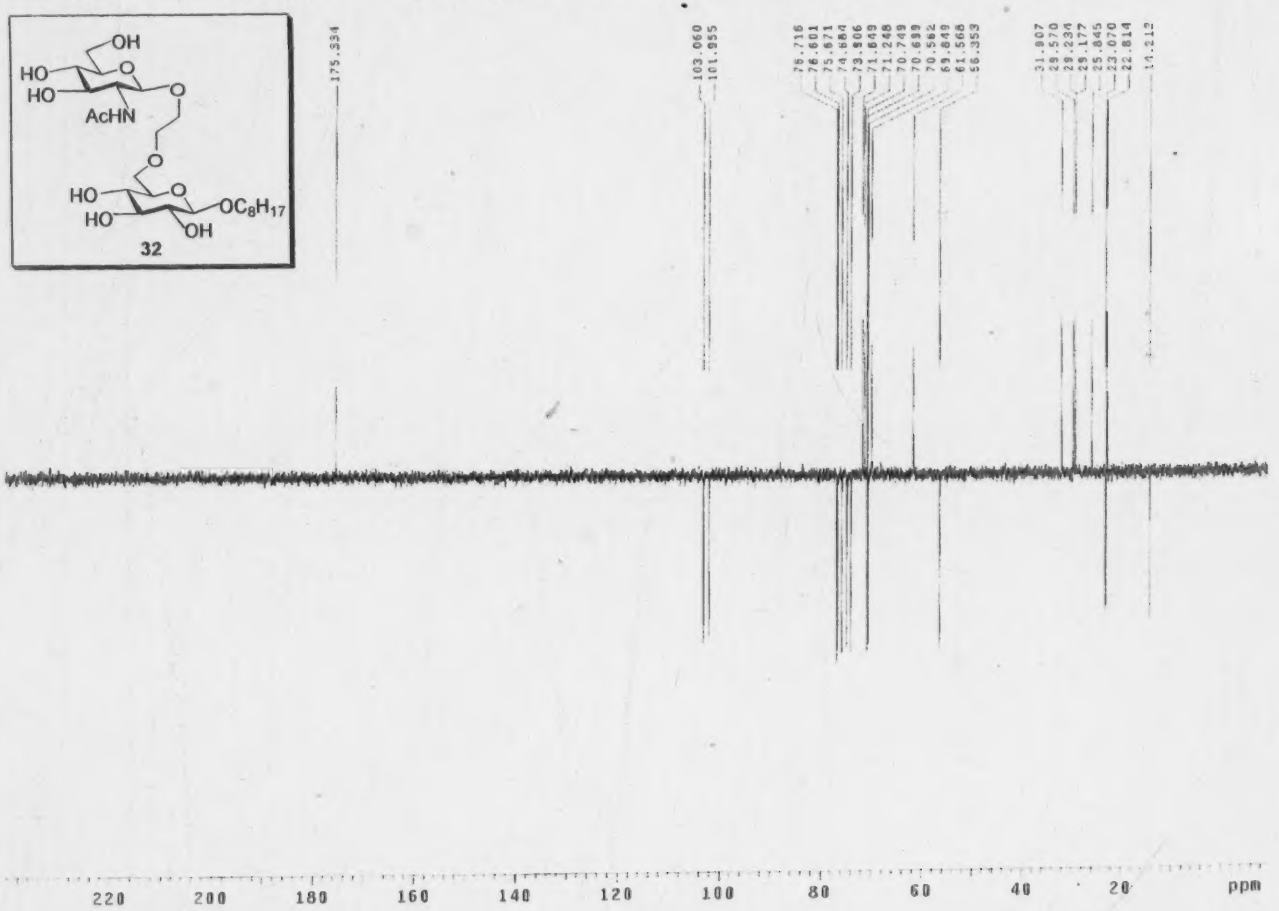


DG-3-77-125 MHz APT in D2O (ref. to 1% acetone @ 31.07 ppm), temp 27.2 C -> actual temp = 27.0 C, sw probe
C & CH2 opposite side of CH & CH3

Pulse Sequence: apt

date:Apr 15 2003 seqfil:apt hz/mm:130.41 sweep width[Hz]:31299 spectrometer:600 file:/mnt/d600/olenmrdata/dristee/DG-3-77-APT-500.fid
acq.time[s]:2.0 relax. time[s]:0.1 dig.res.[Hz/pt]:0.48 # of scans:23384

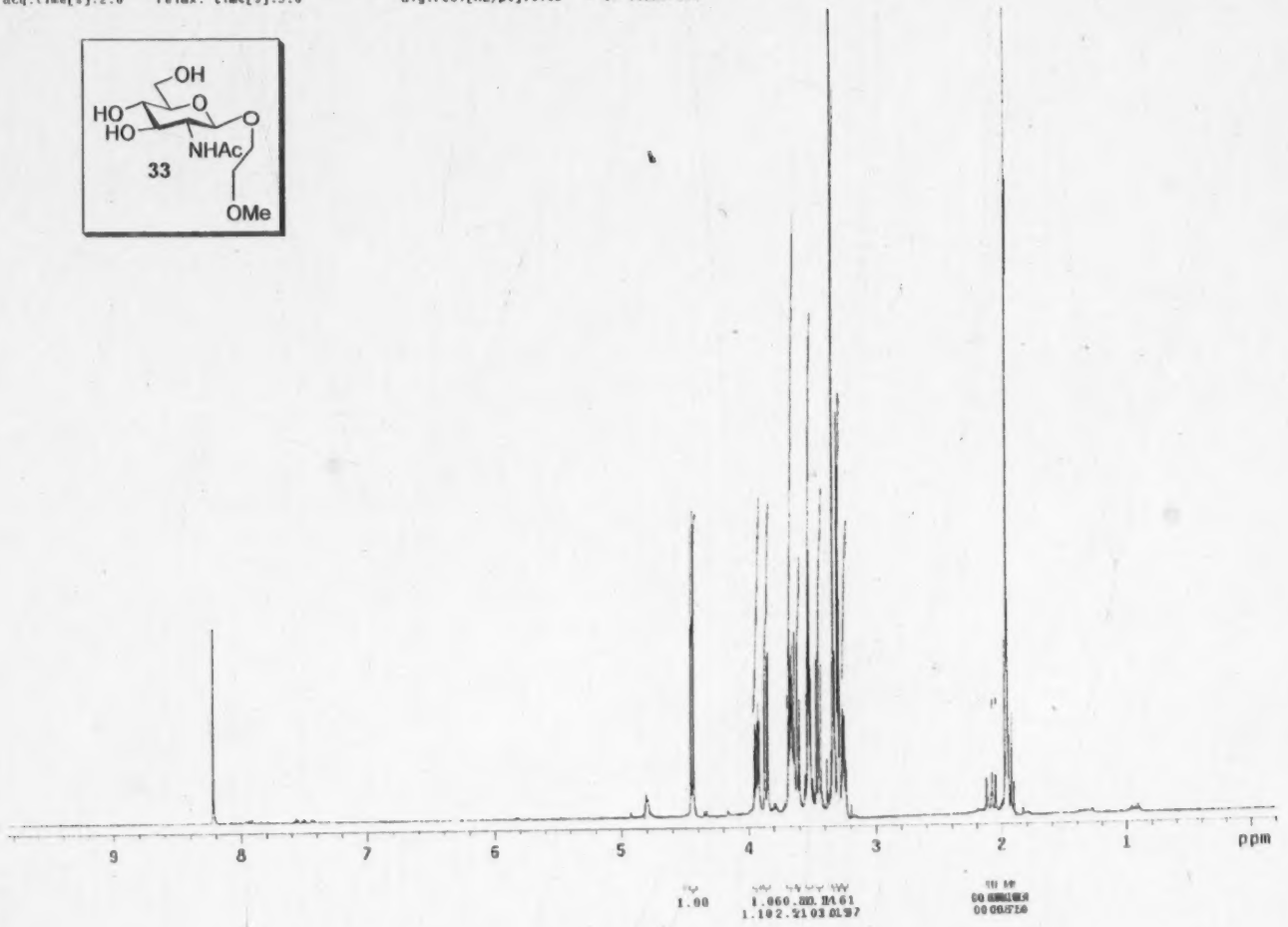
32

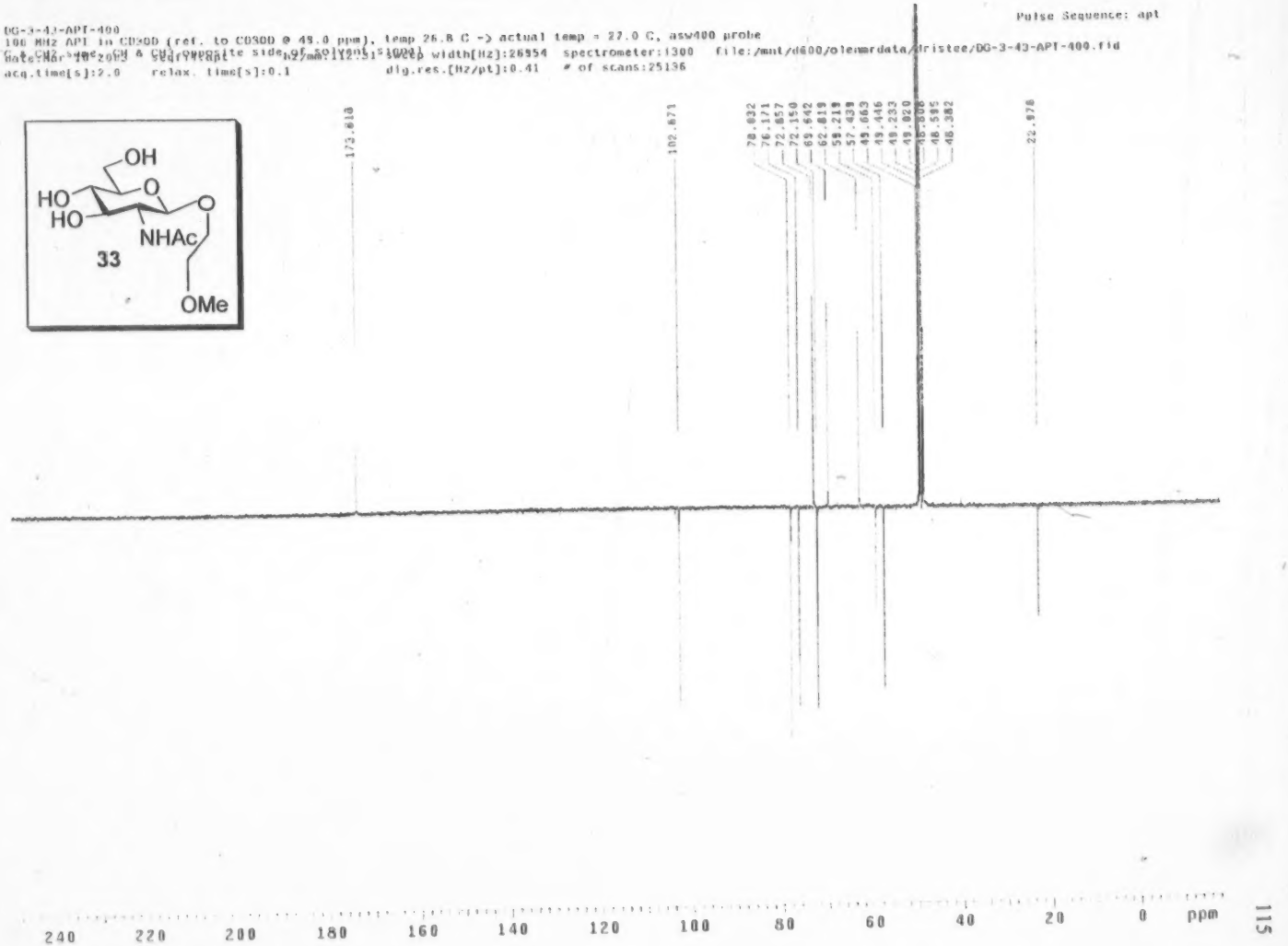


500 MHz 1D in CD3OD (ref. to CD3OD @ 3.30 ppm), temp 27.2 C -> actual temp = 27.0 C, sw500 probe

Pulse Sequence: s2pul

date:Mar 8 2003 seqfil:s2pul hz/mm:20.86 sweep width[Hz]:5006 spectrometer:ibd5 file:exp
acq.time[s]:2.0 relax. time[s]:3.0 dig.res.[Hz/pt]:0.08 # of scans:164




DG-3-43-APT-400
100 MHz APT in CD3OD (ref. to CD3OD @ 49.0 ppm), temp 26.8 C -> actual temp = 27.0 C, asw400 probe
Pulse Sequence: apt
acq.time[s]:2.0 relax. time[s]:0.1 dig.res.[Hz/pt]:0.41 # of scans:25136
OH
HO
HO
O
O
NHAc
33
OMe
173.818
102.871
78.032
76.171
72.857
72.150
69.642
62.819
59.219
57.438
49.663
49.446
49.233
49.020
48.806
48.595
48.382
22.978
240
220
200
180
160
140
120
100
80
60
40
20
0
ppm

7.2 Enzymatic Assay Graphs for 29

7.2.1 Inhibition Graph

Plot of K_{mapp} against different concentrations of inhibitor **29**. A hyperbolic curve is obtained.

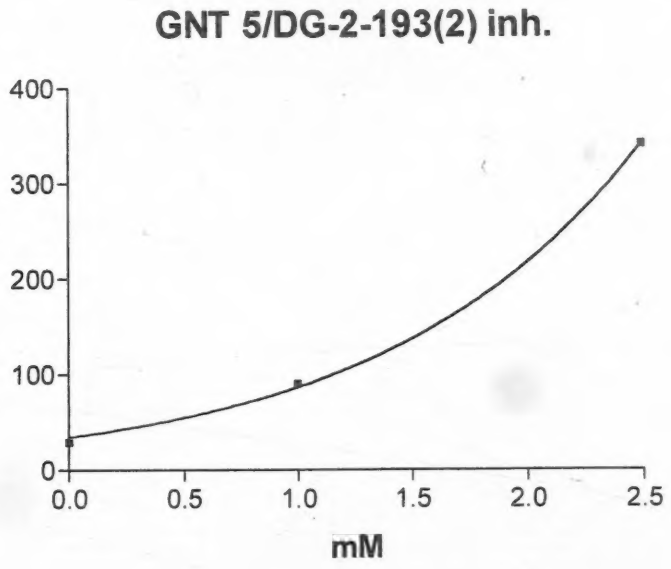


Fig. 48: Plot of K_{mapp} against different concentrations of 29

7.2.2 Activating Property of 29

A plot of 1/V against different concentrations of inhibitor **29** showing that V_{max} increases.

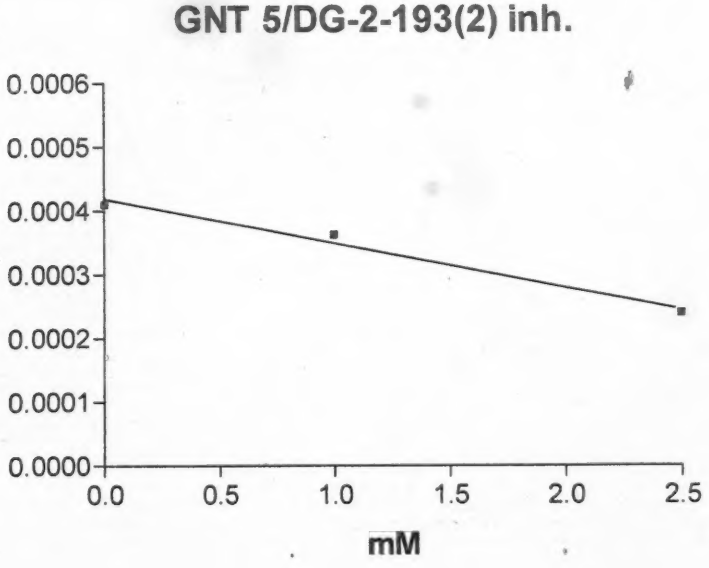


Fig. 49: Plot of 1/V against different concentrations of 29